Gene expression profiling of the canine placenta during normal and antigestagen-induced luteolysis

Nowak, Marta ; Rehrauer, Hubert ; Ay, Serhan S ; Findik, Murat ; Boos, Alois ; Kautz, Ewa ; Kowalewski, Mariusz P

Abstract: The domestic dog is the only domestic animal species that does not produce steroids in the placenta and instead relies on luteal steroids throughout pregnancy. Nevertheless, the canine placenta is highly responsive to steroids, and withdrawal of progesterone (P4) affects the feto-maternal unit, initializing the parturition cascade. Similar effects can be observed during antigestagen-induced abortion. Here, aiming to provide new insights into mechanisms involved in the termination of canine pregnancy, next generation sequencing (NGS, RNA-seq) was applied. Placental transcriptomes derived from natural prepartum and antigestagen-induced abortions were analyzed and compared with fully developed mid-gestation placentas. The contrast "prepartum luteolysis over mid-gestation" revealed 1973 differentially expressed genes (DEG). Terms associated with apoptosis, impairment of vascular function and activation of signaling of several cytokines (e.g., IL-8, IL-3, TGF-) were overrepresented at natural luteolysis. When compared with mid-term, antigestagen treatment revealed 135 highly regulated DEG that were involved in the induced luteolysis and showed similar associations with functional terms and expression patterns as during natural luteolysis. The contrast "antigestagen-induced luteolysis over prepartum luteolysis" revealed that, although similar changes occur in both conditions, they are more pronounced during natural prepartum. Among P4-regulated DEG were those related to immune system and cortisol metabolism. It appears that, besides inducing placental PGF2 output, both natural and induced P4 withdrawal is associated with disruption of the feto-maternal interface, leading to impaired vascular functions, apoptosis and controlled modulation of the immune response. The time-related maturation of the feto-maternal interface needs to be considered because it may be clinically relevant.

DOI: https://doi.org/10.1016/j.ygcen.2019.05.019

Mariusz P (2019). Gene expression profiling of the canine placenta during normal and antigestagen-induced luteolysis. General and Comparative Endocrinology, 282:113194.
DOI: https://doi.org/10.1016/j.ygcen.2019.05.019

General and Comparative Endocrinology 282 (2019) 113194

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Gene expression profiling of the canine placenta during normal and antigestagen-induced luteolysis

Marta Nowak[a], Hubert Rehrauer[b], Serhan S. Ay[c], Murat Findik[c], Alois Boos[a], Ewa Kautz[d], Mariusz P. Kowalewski[a,*]

[a] Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich (UZH), Zurich, Switzerland
[b] Functional Genomics Center Zurich (FGCZ), ETH Zurich/University of Zurich, Zurich, Switzerland
[c] Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, University of Ondokuz Mayis, Samsun, Turkey
[d] Department of Large Animal Diseases with Clinic, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW), Warsaw, Poland

ARTICLE INFO

Keywords:
Dog
Pregnancy
Parturition
Placenta
Transcriptome

ABSTRACT

The domestic dog is the only domestic animal species that does not produce steroids in the placenta and instead relies on luteal steroids throughout pregnancy. Nevertheless, the canine placenta is highly responsive to steroids, and withdrawal of progesterone (P4) affects the feto-maternal unit, initializing the parturition cascade. Similar effects can be observed during antigestagen-induced abortion. Here, aiming to provide new insights into mechanisms involved in the termination of canine pregnancy, next generation sequencing (NGS, RNA-seq) was applied. Placental transcriptomes derived from natural prepartum and antigestagen-induced abortions were analyzed and compared with fully developed mid-gestation placentas. The contrast "prepartum luteolysis over mid-gestation" revealed 1973 differentially expressed genes (DEG). Terms associated with apoptosis, impairment of vascular function and activation of signaling of several cytokines (e.g., IL-8, IL-3, TGF-β) were over-represented at natural luteolysis. When compared with mid-term, antigestagen treatment revealed 135 highly regulated DEG that were involved in the induced luteolysis and showed similar associations with functional terms and expression patterns as during natural luteolysis. The contrast "antigestagen-induced luteolysis over prepartum luteolysis" revealed that, although similar changes occur in both conditions, they are more pronounced during natural prepartum. Among P4-regulated DEG were those related to immune system and cortisol metabolism. It appears that, besides inducing placental PGF2α output, both natural and induced P4 withdrawal is associated with disruption of the feto-maternal interface, leading to impaired vascular functions, apoptosis and controlled modulation of the immune response. The time-related maturation of the feto-maternal interface needs to be considered because it may be clinically relevant.

1. Introduction

Being controlled by species-specific endocrine mechanisms, under physiological conditions pregnancy ends with appropriately timed parturition regulated by complex and synchronized feto-maternal communication, in which the placenta carries a major role (Chaim and Mazor, 1998; Nathanielsz, 1998; Olson, 2003; Lockwood, 2004; Iliodromiti et al., 2012; Kota et al., 2013). Yet, although intensively investigated, the molecular and endocrine mechanisms controlling parturition are still incompletely understood in many species.

Among domestic animal species, sheep belong to those animal models in which the underlying mechanisms have been investigated in the greatest detail, providing knowledge for translational research (Liggins et al., 1973; Magyar et al., 1980; Matthews and Challis, 1996). Consequently, the current concept of the onset of parturition in mammals is greatly influenced by mechanisms described for the ovine species. These show that the signal initializing labor originates from the fetal hypothalamic-pituitary-adrenal (HPA) axis. The maturation of the HPA axis at the end of gestation leads to increased cortisol production by fetal adrenal glands, resulting in activation of placental 17α-hydroxylase (P450C17), and thereby in an enzymatic switch of placental steroidogenesis from the delta 4 to the delta 5 pathway (Anderson et al., 1975). This in turn results in increased placental estrogen synthesis (Steele et al., 1976, Challis et al., 2000). The alteration in the progesterone (P4):estradiol (E2) ratio in favor of the latter sensitizes the uterus to oxytocin (OXT) by increasing expression of its receptor

* Corresponding author at: Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland.
E-mail address: kowalewski@vetanat.uzh.ch (M.P. Kowalewski).

https://doi.org/10.1016/j.ygcen.2019.05.019
Received 9 April 2019; Received in revised form 23 May 2019; Accepted 26 May 2019
Available online 27 May 2019

(OXTR) (Wu et al., 1996). This promotes local synthesis of uterotonic prostaglandin F2α (PGF2α) (Meier et al., 1995).

Similarly, in cattle and pigs, a rise in estrogens is observed prior to parturition (Robertson and King, 1974; Shenavai et al., 2012) and is associated with increased placental activity of steroid sulfatase (STS) which is responsible for production of active estrogens from their biologically inactive sulfoconjugated forms (Schuler et al., 1994; Janszen et al., 1995; Greven et al., 2007). Interestingly, however, in contrast to sheep, in pigs and cattle, both of which depend on luteal P4 for maintenance of pregnancy, active luteolysis is required for initiation of labor, with utero-placental PGF2α acting as the main luteolytic agent (De Rensis et al., 2012; Shenavai et al., 2012).

Departing from the regulatory mechanisms found in domestic animal species in which a systemic withdrawal of P4 is observed prepartum, in humans and guinea pigs elevated P4 levels are still maintained at the time of parturition (Heap and Deanesly, 1966; Brown et al., 2004; Nnamani et al., 2013). In these species, local, i.e., uteroplacental, functional withdrawal of P4 is implicated (Csapo and Pinto-Dantas, 1965; Brown et al., 2004; Zakar and Hertelendy, 2007; Nnamani et al., 2013), and cortisol together with its receptor were indicated as possible antigestagenic factors (Karalis et al., 1996).

The diversity of mechanisms regulating parturition in domestic animal species becomes even wider when the canine species is considered, in which species-specific endocrine mechanisms strongly limit translational research. Thus, the dog is the only domestic animal species devoid of placental steroid production (Hoffmann et al., 1994; Nishiyama et al., 1999). However, although the canine placenta does not produce steroids, it remains highly sensitive to luteal P4, which plays major roles during the maintenance and termination of gestation (Vermeirsch et al., 2000; Kowalewski et al., 2010). Interestingly, the systemic P4 concentrations and secretion profiles are nearly identical in pregnant and non-pregnant bitches for around 60 days of the luteal phase/pregnancy, because there is no luteolysis in the absence of pregnancy in the dog (reviewed in: (Kowalewski, 2014; Kowalewski, 2018) Shortly before parturition, a rapid drop in P4 occurs in pregnant individuals signaling the onset of parturition (Nohr et al., 1993; Hoffmann et al., 1994; Concannon, 2011). This prepartum drop in P4 is concomitant with elevated PGF2α levels in the maternal circulation, indicating the role of PGF2α during luteolysis and/or parturition (Nohr et al., 1993; Hoffmann et al., 1994; Concannon, 2011). In contrast to other domestic animal species, there is no pregnancy- and/or parturition-associated increase in estrogens in the dog (Hoffmann et al., 1994). In fact, E2 levels decrease together with P4, further indicating the luteal origin of E2. The elevated levels of cortisol in maternal serum prior to delivery are inconsistent, and were related to maternal stress rather than to the active induction of parturition (Hoffmann et al., 1994). Regardless, increased circulating cortisol levels in maternal peripheral plasma do not appear to be compulsory for initiation of the parturition cascade in the dog (Hoffmann et al., 1994). The placental feto-maternal communication between maternal stroma-derived decidual cells and fetal trophoblast seems to be involved in underlying regulatory mechanisms initiating parturition in the dog (Kowalewski et al., 2010). Thus, in the canine placenta, only decidual cells carry the nuclear P4 receptor, PGR (Vermeirsch et al., 2000; Kowalewski et al., 2010). The prepartum increase in PGF2α appears to originate predominantly in the fetal part of the placenta, i.e., in the trophoblast, in which the prostaglandin synthesizing machinery is strongly expressed during prepartum luteolysis (Kowalewski et al., 2010; Gram et al., 2013, 2014). Blocking the functionality of PGR by treatment with an antigestagen, such as aglepristone, leads to similar effects as observed during natural withdrawal of P4, i.e., induction of placental PGF2α synthesis and initiation of the prepartum signaling cascade (Nohr et al., 1993; Kowalewski et al., 2010).

Here, given the great importance of the placental feto-maternal communication in the initiation of canine labor, we aimed to provide new insights into the molecular regulation of the canine placenta during termination of pregnancy. To assess differences in global gene expression in the canine placenta, a transcriptomic approach applying next generation sequencing (NGS, RNA-Seq) was employed. Placental samples collected at natural prepartum luteolysis were compared with those samples obtained from dogs at the mid-pregnancy stage. Furthermore, in order to better understand the involvement of P4 and PGR-dependent downstream regulatory mechanisms during initiation of parturition in the dog, samples were included derived from dogs in which luteolysis/abortion was induced with the antigestagen aglepristone at mid-pregnancy. To identify differences in molecular events occurring in the placenta prior to natural parturition and/or abortion, placental transcriptomes of both groups were compared. With this approach, the aim was to acquire new information that could be translated into clinical and breeding practices for more accurate patient management.

To our knowledge, this is the first study providing a global transcriptome analysis of the canine placenta to highlight differences between parturition and abortion, and also adding new insights into molecular regulation of the luteolytic cascade in the dog.

2. Materials and methods

2.1. Animals and tissue sampling

Placentae from thirteen (n = 13) clinically healthy, cross-breed bitches (aged 2–8 years) were included in this study. Animals were assigned to the following experimental groups: 1) mid-gestation (Group 1: days 35–40 of pregnancy; n = 5); 2) natural prepartum luteolysis (Group 2: n = 3); 3) antigestagen-induced luteolysis (Group 3: n = 5). The discovery samples for the RNA-Seq experiment consisted of 9 placentas (n = 3/per group), while TaqMan RT-qPCR validation experiments were performed using all available samples (n = 13 samples, grouped as stated above). All dogs used for the study were subjected to routine ovariohysterectomy (OVH). Dogs were mated 2 days after ovulation, which was determined by vaginal cytology and measurements of serum P4 concentrations (> 5 ng/ml) by radioimmunoassay as described previously (Hoffmann et al., 1973; Kowalewski et al., 2009). The day of mating represented Day 0 of gestation. The time of natural prepartum luteolysis (Group 2) was ascertained by measurements of serum P4 at 6 h intervals beginning on day 58 of pregnancy. Under physiological conditions, parturition in dogs occurs 12–42 h following the luteolytic P4 drop (Concannon et al., 1977, 1989). Therefore, in Group 2, when P4 levels dropped below 3 ng/ml in three consecutive measurements indicating prepartum luteolysis, OVH was performed and tissue samples were collected. All P4 concentrations were reported previously (Kowalewski et al., 2009). The number of animals included in the prepartum luteolysis group was limited due to formal restrictions by the ethics committee, resulting from the late timing of sample collection (prior to the first clinical signs of parturition) and possible danger to fetuses. Additionally, abortion was induced in bitches at mid-pregnancy, day 40–45 (Group 3) using a P4-receptor (PGR) blocker, aglepristone (Alizine®, Virbac, Bad Oldesloe, Germany) administrated subcutaneously at a dose of 10 mg/kg bw, twice at 24 h interval. OVH and tissue collections were done 24 h after the second treatment.

Following surgery, placentae were separated from uteri, rinsed with phosphate-buffered saline (PBS) and immersed in RNAlater® for 24 h at +4 °C. After 24 h, tissue material was stored at −80 °C until RNA isolation.

All animal experiments and use of tissue samples were in accordance with animal welfare legislation and were approved by the respective authorities of the Justus Liebig University, Giessen, Germany (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c-20/15c GI 18,14) and the Ondokuz Mayıs University Samsun, Turkey (Animal Experiments Local Ethics Committee, HADYEK, permit no. 2015/82).

Table 1
List of primers and probes used for real time TaqMan qPCR (RT-qPCR).

Gene	Gene name	Accession number	Sequence/Product no.	Amplicon length
IGF-1	insulin-like growth factor I	NM_001313855	Cf02627846_m1	104 bp
IGFBP3	insulin-like growth factor binding protein 3	XM_003639557	Cf02655026_g1	85 bp
IGFBP4	insulin-like growth factor binding protein 4	XM_845091	Cf02656699_m1	98 bp
IGFBP5	insulin-like growth factor binding protein 5	XM_847792	Cf02691124_s1	89 bp
INHBA	inhibin, subunit beta A	XM_540364	Cf02696654_u1	157 bp
INHBB	inhibin, subunit beta B	XM_005631933	Cf02696662_g1	83 bp
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	XM_005620822	Cf02690463_s1	82 bp
TF	transferrin	XM_005634449	Cf02630418_m1	68 bp
TGFB2	transforming growth factor, beta 2	XM_545713	Cf02676764_m1	144 bp
TGFB3	transforming growth factor, beta 3	XM_849026	Cf02683792_s1	59 bp
TGFBR1	transforming growth factor, beta receptor 1	XM_014117881	Cf02687913_m1	110 bp
TGFBR2	transforming growth factor, beta receptor 2	XM_014106674	Cf02625160_m1	57 bp
CXCR2	interleukin 8 receptor, beta	NM_001003151	Cf04419503_u1	84 bp
ICAM1	intercellular adhesion molecule 1	NM_001003291	Cf02690470_u1	124 bp
ADIPOQ (APM1)	adiponectin, C1Q and collagen domain containing	XM_005639713	Cf02703591_s1	189 bp
E2F4	E2F transcription factor 4	XM_848882	Cf02668600_m1	142 bp
RANBP3	RAN binding protein 3	MK514274		94 bp
forward			5′-ACA AGG CCA GTG AGA AGA GCA T-3′	
reverse			5′-GTG TCC TTG GAA CTT GCT GAG A-3′	
probe			5′-CCA GGG CGT GAA GGT TTT CTT-3′	
IL-8 (CXCL8)	interleukin 8	NM_001003200		114 bp
forward			5′-CCA CAC CTT TCC ATC CCA AA-3′	
reverse			5′-CCA GGC ACA CCT CAT TTC CA-3′	
probe			5′-CTG AGA GTG ATT GAC AGT GGC CCA CAT TGT-3′	

2.2. RNA isolation and purification

Total RNA was isolated from separated placental samples using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. The RNA purity and quantity were assessed with a NanoDrop 2000C® spectrophotometer (Thermo Fischer Scientific AG, Reinach, Switzerland). Additionally, RNA samples subjected to deep sequencing were purified using a RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Finally, RNA integrity was assessed with the Agilent 2200 TapeStation System. RNA integrity numbers (RIN) ranged between 7.4 and 9.8.

2.3. RNA sequencing

RNA-Seq experiments were performed in accordance with our previously published protocol (Zatta et al., 2017). Briefly, the quality and quantity of purified total RNA were determined with a Qubit® (1.0) fluorometer (Life Technologies, City, California, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Each RNA sample (100–1000 ng) was used in a TruSeq RNA Sample Prep Kit v2 (Illumina, Inc., City, California, USA) protocol for library preparation.

With this, samples were poly-A enriched and underwent reverse-transcription to cDNA. Processing of cDNA samples included the following steps: fragmentation, polyadenylation and ligation of TruSeq adapters. Fragments containing adapters on both ends were selectively amplified with PCR. Finally, the quality and quantity of enriched libraries were evaluated using the Qubit® (1.0) fluorometer and the Caliper GX LabChip® GX (Caliper Life Sciences, Inc., Hopkinton, MA, USA). Following normalization to 10 nM, libraries were pooled. From these, 10 pM was used for clustering in a TruSeq SR Cluster Kit v4-cBot-HS (Illumina, Inc.). Sequencing was performed on the Illumina HiSeq4000 single end 125 bp using the TruSeq SBS Kit v4-HS (Illumina, Inc.). The data generated herein have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE126031.

2.4. Data evaluation

The raw data, i.e., fastq files delivered by the Illumina HiSeq4000 sequencing system were analyzed using SUSHI (Hatakeyama et al., 2016; Qi et al., 2017), a framework for analysis of NGS data, developed at the Functional Genomics Center Zurich (FGCZ). Briefly, after quality control, the reads were aligned to a reference genome (the Ensembl genome build CanFam3.1; www.ensembl.org/Canis_familiaris/Info/Index) and normalized. The gene was considered detected if it had an average count of at least 10 reads in at least one group of replicates. The generalized linear model approach of the Bioconductor package DESeq2 (Love et al., 2014) served to assess differential expression between experimental groups and was used as previously reported (Zatta et al., 2017). Significance of differential expression was assessed using the Wald test for the coefficients of the fitted model. A more detailed description can be found in the documentation of the Bioconductor package DESeq2 (Love et al., 2014). The Benjamini-Hochberg algorithm, which computes False Discovery Rate (FDR, adjusted P-value), was applied for correction of multiple testing. Differentially expressed genes (DEG) were identified for selected contrasts, i.e., pairwise comparisons. The thresholds of P-value < 0.01 and adjusted P-value < 0.1 (i.e., FDR $< 10\%$) were applied. Complete DEG lists for each comparison, as provided in Supplemental Files 1–3, served for further downstream analyses. Next, DEG were functionally characterized and enrichment scores of over-represented functional categories were calculated using PANTHER software (pantherdb.org, (Mi et al., 2017)). The web-based software Ingenuity Pathway Analysis (IPA®, Qiagen, Redwood City, California, USA) was used to determine most significantly affected pathways and the involved upstream regulators. Additionally, the Bioconductor package *goseq* (Young et al., 2010) and an integrative web-based and mobile software application Enrichr (Chen et al., 2013) were applied to support and corroborate the results. The ClueGO v2.2.3 (Bindea et al., 2009), an application of the open source bioinformatics software platform Cytoscape v3.5.1 (Shannon et al., 2003), was used to visualize enriched functional biological networks for the pairwise comparison: "prepartum luteolysis over mid-gestation". In addition, Venn diagrams were generated to show overlap of DEG between selected contrasts. The input data for Venn diagrams included an additional fold-change threshold of up- ($\log2\text{Ratio} \geq 0.5$) and down- ($\log2\text{Ratio} \leq -0.5$) regulation.

2.5. Expression of selected target genes by semi-quantitative real time TaqMan qPCR (RT-qPCR)

In order to validate generated RNA-Seq data, the expression of sixteen (16) selected candidate genes was evaluated. A complete list of

primers used, TaqMan-probes and pre-designed TaqMan systems is provided in Table 1. Based on published canine CDS sequences, gene-specific primers and probes labeled with 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (TAMRA) were designed using Primer Express Software version 2.0 (Applied Biosystems, by Thermo Fisher Scientific, Carlsbad, CA, USA) and ordered from Microsynth, Balgach, Switzerland. The efficiencies of PCR assays were determined by the CT slope method ensuring approximately 100% reaction efficiency (Kowalewski et al., 2011). The pre-designed TaqMan systems were ordered from Applied Biosystems.

The validation experiments were performed using all available (n = 13) placental samples. For each sample, 10 ng of RNA were used for downstream pre-amplification procedures. In order to eliminate genomic DNA contamination, DNase treatment was performed using RQ1 RNase-free DNase (Promega, Duebendorf, Switzerland). The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription (RT). The RT-reaction was followed by cDNA pre-amplification, which was done using the TaqMan PreAmp Master Mix Kit (Applied Biosystems) according to the manufacturer's protocol and as described previously (Nowak et al., 2017). RT-qPCR experiments were done following our previously published protocols (Kowalewski et al., 2006, 2011). In brief, an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems) was used for all semi-quantitative analyses of target gene mRNA expression. Each sample was run in duplicates in 96-well optical plates (Applied Biosystems). Autoclaved water and the so-called minus-RT controls (i.e., samples treated with DNase but not subjected to RT) instead of cDNA were used as negative controls. The conditions for amplification were set as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles for 15 s at 95 °C and 1 min at 60 °C, each.

2.6. Semi-quantification and normalization of target gene expression

The semi-quantification of target gene expression was done by applying the comparative Ct method (ΔΔ Ct method) according to the ABI 7500 Fast Real-Time PCR System protocol and as described previously (Kowalewski et al., 2006, 2011). RT-qPCR data were normalized according to two reference genes: *E2F4*, a transcription factor from the E2F family, and *RANBP3*, a gene encoding for a protein important for nucleocytoplasmic transport. Both reference genes were chosen from RNA-Seq datasets presented herein and were defined as non-differentially and stably expressed genes in all examined samples. The stability of their expression was evaluated using an on-line tool RefFinder (http://150.216.56.64/referencegene.php#, (Xie et al., 2012)) and NormFinder software (Andersen et al., 2004). RT-qPCR data were logarithmically transformed and are presented as geometric means (Xg) ± geometric standard deviation (SD). An unpaired, two-tailed Student's *t*-test was done using GraphPad 3.06 (GraphPad Software Inc., San Diego, CA, USA) to compare the expression of target genes between respective groups. Changes were considered significant if $P < 0.05$.

3. Results

3.1. Differential expression analysis (pairwise comparisons)

Differential expression analysis was done for selected experimental conditions using the DESeq2 package for Bioconductor. Only features (i.e., genes) with counts above the threshold of 10 reads per gene were considered detected. Genes were assumed to be differentially expressed (DEG = differentially expressed genes) if $P < 0.01$ and FDR < 0.1. Table 2 presents a summary of the results for all tested contrasts. Detailed lists of DEG from respective pairwise comparisons are provided as Supplemental Files 1–3.

3.1.1. Contrast "prepartum luteolysis over mid-gestation"

We detected 13,769 genes reaching the threshold of at least 10 reads per gene. From these, 1973 genes were differentially expressed between the two groups: 1034 DEG were up- and 939 were down-regulated at prepartum luteolysis when compared to mid-gestation.

3.1.2. Contrast "antigestagen over mid-gestation"

For this contrast, 13,884 features were detected. Of the 412 DEG initially selected based on the $P < 0.01$ criterium, 135 genes passed the FDR < 0.1 correction: of these 80 genes were up- and 55 were down-regulated in the induced luteolysis group.

3.1.3. Contrast "antigestagen over prepartum luteolysis"

For this contrast, 13,752 genes were detected. Of the 1509 DEG, 810 were more highly expressed in the antigestagen-induced luteolysis group, and 699 were more highly expressed in the prepartum luteolysis group.

3.2. Functional annotations

In the next step, DEG from the respective pairwise comparisons were functionally classified according to Gene Ontology (GO) terms. Enrichment scores were calculated for each term. All GO analyses were done using PANTHER software, and the *goseq* package was used to corroborate the results. The GO analysis covers three main functional domains: biological process (BP), cellular compartment (CC) and molecular function (MF). Lists of representative genes involved in respective enriched GO terms in each contrast are presented in Supplemental File 4.

3.2.1. Contrast "prepartum luteolysis over mid-gestation"

Genes more highly expressed at prepartum luteolysis were strongly associated with the following selected GO terms (Fig. 1A, Supplemental File 4): negative regulation of endothelial cell proliferation (P = 4.90E−06), negative regulation of cell-matrix adhesion (P = 8.04E−04), positive regulation of cholesterol efflux (P = 1.34E−04), positive regulation of insulin-like growth factor (IGF) receptor signaling pathway (P = 3.46E−04), positive regulation of cholesterol transport (P = 5.91E−04), negative regulation of cell migration (P = 5.35E−08), negative regulation of cell adhesion (P = 9.99E−05), response to extracellular stimulus (P = 4.21E−05), enzyme linked receptor protein signaling pathway (P = 1.83E−08), positive regulation of apoptotic process (P = 3.15E−04) and intracellular signal transduction (P = 1.29E−03).

Functional terms overrepresented in the mid-pregnant group included predominantly (Fig. 1A, Supplemental File 4): initiation of DNA replication (P = 1.54E−11), positive regulation of cytokinesis (P = 4.47E−05), DNA replication (P = 1.17E−17), spindle assembly (P = 2.90E−08), mitotic cell cycle process (P = 6.02E−31), positive regulation of cell division (P = 5.86E−05), chromosome segregation (P = 2.52E−18) and RNA processing (P = 6.87E−05).

Additionally, the functional classification of genes more highly expressed in either the prepartum luteolysis or mid-gestation group was further evaluated in the "cellular process" category, and compared to standard categorization of the canine reference genome (Fig. 1B). Thus, compared with the reference genome, those genes upregulated at mid-gestation were strongly enriched in the cell cycle (31.1%) and chromosome segregation (9.1%) sub-categories, which were not that strongly pronounced at prepartum (9.9% and 1.3%, respectively). This was also higher compared with the reference genome (16.9% and 2.1%, from cell cycle and chromosome segregation, respectively). On the other hand, displaying 61.6% of genes associated with cell communication, genes involved in this sub-category were increased during prepartum luteolysis and represented 70.8%. This proportion was also strongly increased compared with the 37% of genes associated with cell communication during mid-gestation.

3.2.2. Contrast "antigestagen over mid-gestation"

Genes more highly represented during antigestagen-induced

Table 2
Summary of RNA-Seq results for all contrasts investigated in the study. Feature counts including DEG (differentially expressed genes) numbers are presented.

Analysis	Prepartum luteolysis over mid-gestation	Antigestagen over mid-gestation	Antigestagen over prepartum luteolysis
Genes Total (P-value < 0.01, FDR < 0.1)	1973	135	1509
Genes Up	1034	80	810
Genes Down	939	55	699
Number of genes with counts above threshold	13,769	13,884	13,752

luteolysis significantly enriched GO terms associated with (Fig. 2A, Supplemental File 4): negative regulation of cell-substrate adhesion (P = 2.04E−05), blood coagulation (2.64E−05) and response to wounding (3.59E−05). Higher functional variation and, therefore, no significant terms were found for the 55 genes more highly expressed in the mid-gestation group. Next, all 135 DEG were analyzed in the "cellular process" category compared with the reference genome, revealing an enrichment of cell communication sub-category (75% versus 61.5%) and lower association with cell cycle (6.8%, 16.9%) (Fig. 2B).

3.2.3. Contrast "antigestagen over prepartum luteolysis"

In this contrast, we found the following terms were more highly represented during induced luteolysis (Fig. 3, Supplemental File 4): DNA replication initiation (P = 3.19E−10), positive regulation of cytokinesis (P = 1.31E−04), positive regulation of cell division (P = 4.95E−04), positive regulation of cell cycle process (P = 7.74E−12), extracellular matrix organization (P = 1.45E−04) and sulfur amino acid biosynthetic process (P = 4.42E−04). More highly represented gene ontologies during prepartum luteolysis were

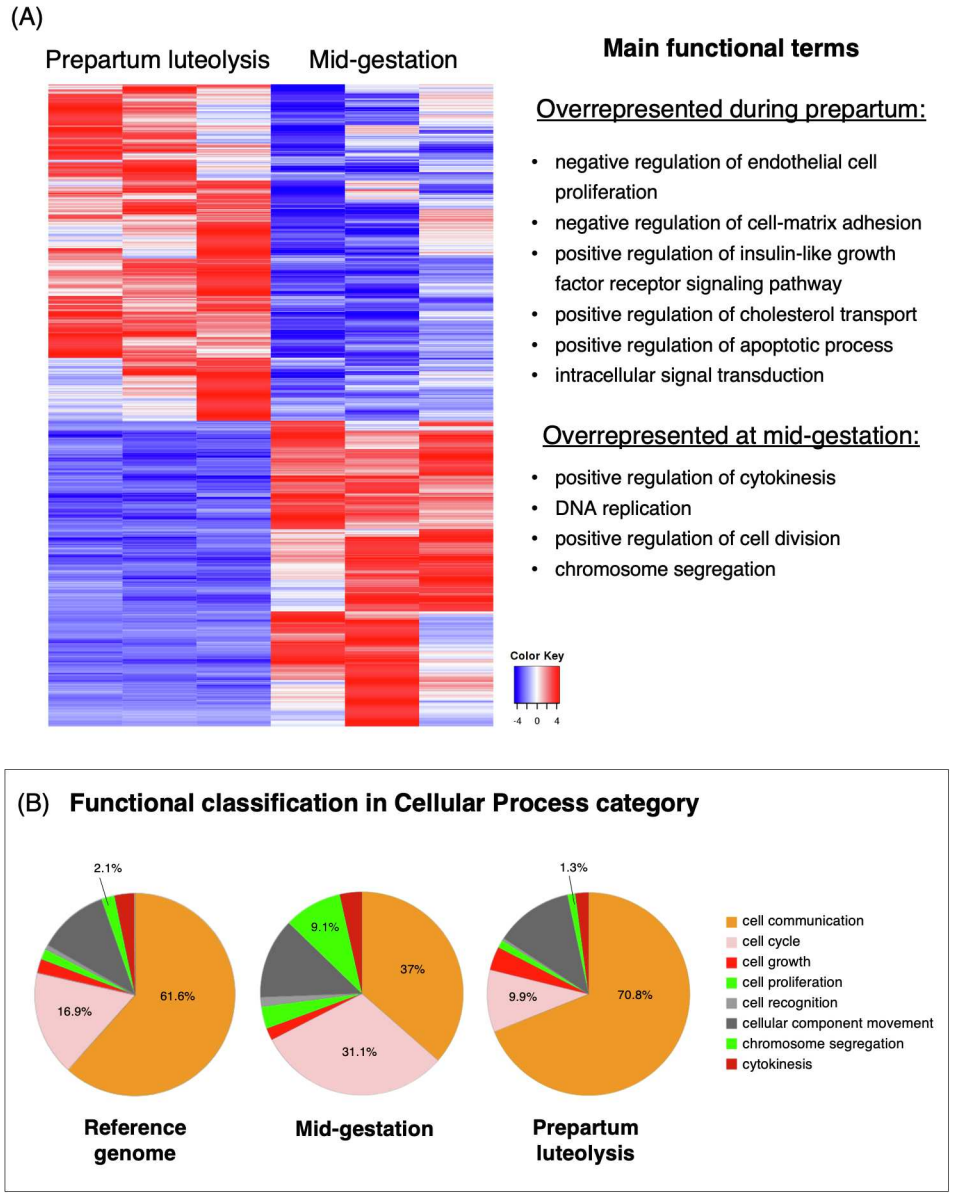

Fig. 1. (A) Heatmap of 1973 differentially expressed genes (DEG) of the contrast "prepartum luteolysis over mid-gestation". Colors red to blue indicate a gradient of high to low expression of each gene relative to its average expression. 1034 genes were more and 939 were less highly expressed in the prepartum luteolysis group (P < 0.01, FDR < 0.1). The main overrepresented functional GO terms in each of the groups are listed (statistical details are provided in the text and Supplemental File 4). The entire list of DEG is provided as Supplemental File 1. (B) The functional classification of genes more highly expressed in the prepartum luteolysis or mid-gestation groups in the "cellular process" category, compared with standard categorization of the reference genome. During prepartum luteolysis, upregulated genes were associated mainly with "cell communication", whereas genes more highly expressed in the mid-gestation group were more strongly associated with "cell cycle" sub-category.

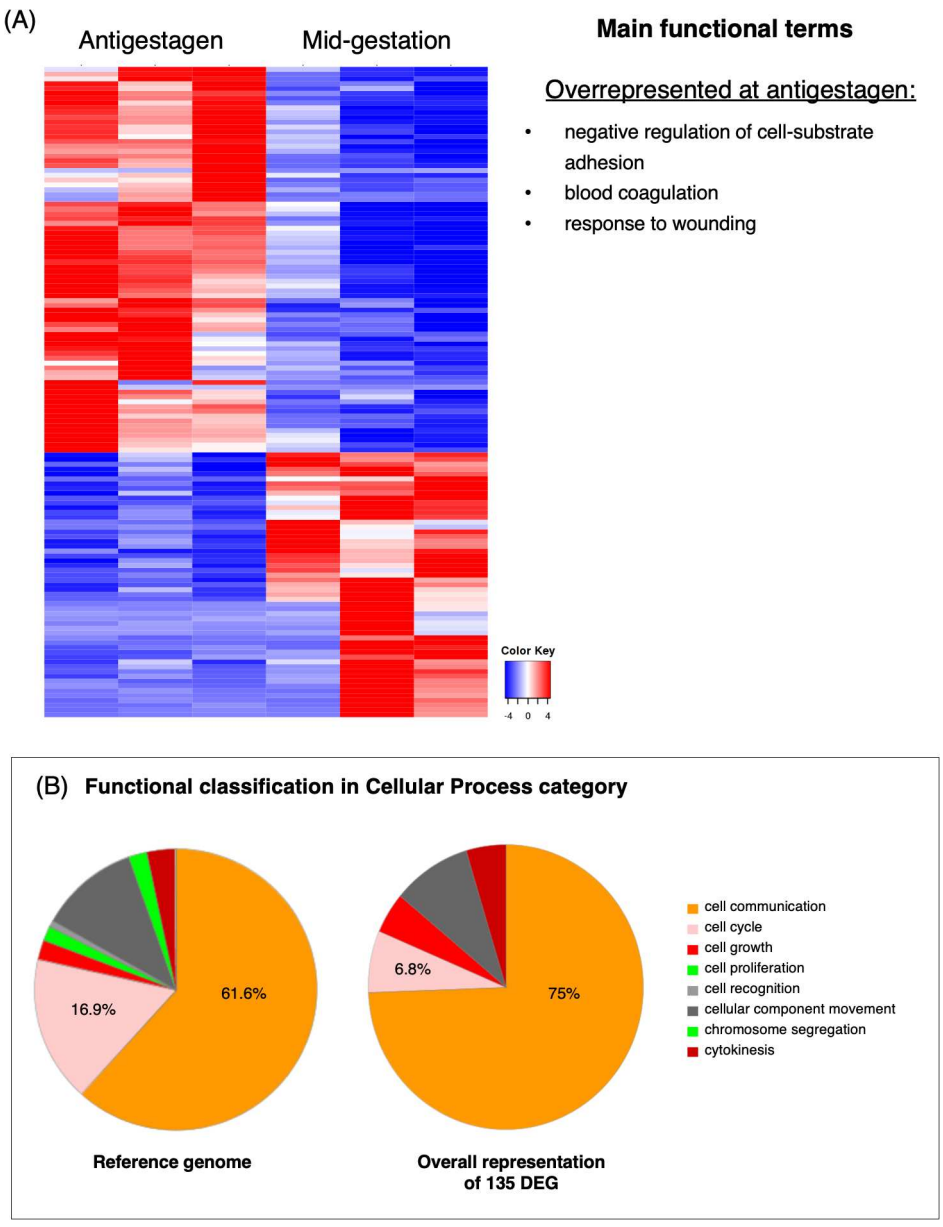

Fig. 2. (A) Heatmap of 135 differentially expressed genes (DEG) of the contrast "antigestagen over mid-gestation". Colors red to blue indicate a gradient of high to low expression of each gene relative to its average expression. 80 genes were more and 55 were less highly expressed during induced luteolysis ($P < 0.01$, FDR < 0.1). The main overrepresented functional GO terms are listed (statistical details are provided in the text and Supplemental File 4). The entire list of DEG is provided as Supplemental File 2. (B) The overall functional classification of all 135 DEG in the "cellular process" category, in comparison with standard categorization of the reference genome. "Cell communication" sub-category was more enriched and "cell cycle" was less enriched when compared to distribution of the reference genome.

significantly associated with these terms: response to progesterone ($P = 9.02E-04$), negative regulation of angiogenesis ($P = 7.88E-03$), regulation of cytokine production ($P = 7.58E-04$), positive regulation of protein kinase C activity ($P = 1.13E-03$), negative regulation of cell adhesion mediated by integrin ($P = 2.10E-04$), positive regulation of insulin-like growth factor (IGF) receptor signaling pathway ($P = 6.61E-04$), positive regulation of cholesterol efflux ($P = 1.56E-03$), positive regulation of glycolytic process ($P = 1.77E-03$), positive regulation of blood coagulation ($P = 7.90E-04$), and positive regulation of immune system process ($P = 1.27E-03$).

3.3. Functional networks by Cytoscape

Using the ClueGO plug-in of the open source platform Cytoscape, we functionally grouped and visualized gene ontology networks enriched by upregulated (Fig. 4A) and downregulated genes (Fig. 4B) from the contrast "prepartum luteolysis over mid-gestation". For input, respective lists of DEG (Supplemental File 1) were used. Networks and up to 10 top regulated involved genes are listed in Supplemental File 4.

The most represented functional networks during prepartum luteolysis were mainly associated with cell communication and motility, and with modulation of vascular function and metabolic processes, and included: vasculature development, cell motility and migration, lipid metabolic process, regulation of cell communication and cellular response to stimulus. In contrast, among networks more highly represented during mid-gestation most prevalent were those related to proliferative activities: DNA metabolic process, chromosome segregation, cytoskeleton organization, cell division and cell cycle.

3.4. IPA: ingenuity pathway analysis

3.4.1. Pathways

To determine the most significantly affected signaling pathways, lists of DEG ($P < 0.01$, FDR < 0.1) from each comparison were used as input for Ingenuity Pathway Analysis Software (IPA, Qiagen). A comprehensive list of pathways found, the representative top 10 involved genes, as well as statistical details are presented in Supplemental File 4.

For the contrast "prepartum luteolysis over mid-gestation", among the most markedly enriched pathways predicted to be activated during

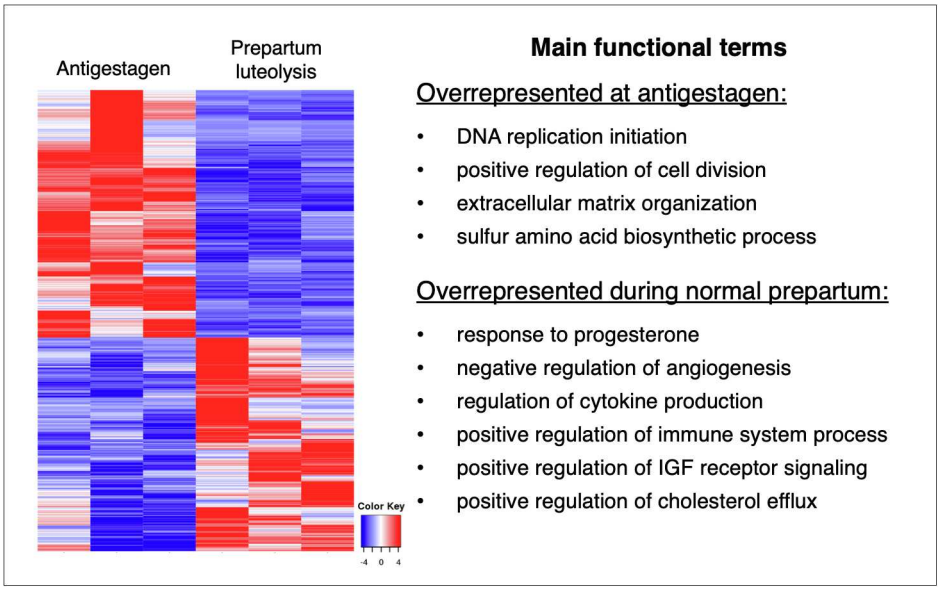

Fig. 3. Heatmap of 1509 differentially expressed genes (DEG) of the contrast "antigestagen over prepartum luteolysis". Colors red to blue indicate a gradient of high to low expression of each gene relative to its average expression. 810 genes were more highly expressed in the antigestagen-induced luteolysis group, whereas 699 were more highly expressed during prepartum luteolysis ($P < 0.01$, FDR < 0.1). The main over-represented functional GO terms in each of the groups are listed (statistical details are provided in the text and Supplemental File 4). The entire list of DEG is provided as Supplemental File 3.

prepartum were those associated with (Fig. 5, Supplemental File 4): immune response (IL-8 signaling, P = 2.75E−05; NF-κB signaling, P = 5.89E−03; TGF-β signaling, P = 1.58E−02; and IL-3 signaling, P = 2.40E−02), regulation of vascular function (IL-8 signaling, P = 2.75E−05; VEGF signaling, P = 2.75E−03; and angiopoietin signaling, P = 5.50E−03), IGF-1 signaling (P = 3.80E−03) and G-protein coupled receptor signaling (Gαq signaling, P = 3.31E−02; protein kinase A (PKA) signaling, P = 3.98E−02).

Among significant pathways that were predicted to be inhibited in the prepartum luteolysis group we found: mitotic roles of polo-like kinase (P = 1.66E−06), cyclins and cell cycle regulation (P = 2.69E−04), leukocyte extravasation signaling (P = 1.02E−03) and PPAR signaling (P = 2.69E−02).

Regarding the contrast "antigestagen over mid-gestation", among the most highly enriched pathways were (Fig. 6, Supplemental File 4): inhibition of matrix metalloproteases (P = 6.92E−05), intrinsic prothrombin activation pathway (P = 9.33E−05), coagulation system (P = 1.66E−02), granulocyte adhesion and diapedesis (P = 1.91E−02), leukocyte extravasation signaling (P = 3.24E−02), HIF1α signaling (P = 2.95E−02), IL-8 signaling (P = 2.57E−02) and glucocorticoid receptor signaling (P = 4.37E−02).

Finally, the most significantly affected pathways in the comparison "antigestagen over prepartum luteolysis" (Fig. 7, Supplemental File 4) were similar to those observed for the contrast "prepartum luteolysis over mid-gestation" and were represented by, e.g.,: IL-8 signaling (P = 6.46E−06), TGF-β signaling (P = 5.75E−04), Gαq signaling (P = 2.14E−03), NF-κB signaling (P = 8.51E−03), endothelin-1 signaling (P = 2.4E−02) and PKA signaling (P = 1.26E−02), but the activation of most of these pathways was potentiated more during prepartum than antigestagen-induced luteolysis.

3.4.2. Upstream regulators and potential P4-regulated genes

Additionally, IPA® software was used to identify upstream regulators, i.e., molecules such as transcriptional factors and cytokines, which can affect the expression of a given population of genes in a specific manner. DEG from the contrasts "prepartum luteolysis over mid-gestation" and "antigestagen over mid-gestation" were found to be affected upstream by: P4, PGR, TNF, dexamethasone, TGF-β, IGF-1 and PPARγ (for statistical details see Supplemental File 4). In the contrast "antigestagen over prepartum luteolysis", among the most represented upstream regulators, we identified TGF-β, dexamethasone, P4 and PGR. The first two molecules, i.e., TGF-β and dexamethasone appeared to be more enriched during prepartum luteolysis.

Further, because withdrawal of P4 is crucial for initiation of parturition and, as presented above, this hormone was identified as an important upstream regulating molecule in the analyzed datasets, we used IPA® to examine expression of genes potentially affected by P4, i.e., P4-regulated genes. Thus, for the comparison "prepartum luteolysis over mid-gestation" 127 genes were found to be potentially influenced by P4 (Fig. 8A). Among them were, i.a.: matrix metalloproteases (*MMP1, MMP2, MMP9, MMP13*), *ICAM1*, members of the *IGF-* (*IGF1, IGFBP3, IGFBP5*) and *TGF-β*-systems (*TGFβ3, TGFBR1*), *ADIPOQ, PRLR, VEGFA*, *PTGS2* and *HSD11B2*. Regarding the comparison "antigestagen over mid-gestation", 14 genes were identified as potentially affected by P4 (Fig. 8B). Nine of these genes, *ERRFI1, F2RL1, Gstt1, HSD11B2, IGFBP5, LPL, MMP1, PARM1* and *SPHK1*, were found in both datasets and their expression was regulated in the same manner, i.e., either up- or down-regulated.

3.5. Venn diagrams

To reveal similarities and differences in the placental transcriptome from natural, i.e., prepartum, vs. induced luteolysis, the intersections of DEG from contrasts "prepartum luteolysis over mid-gestation" vs. "antigestagen over mid-gestation" were visualized using Venn diagrams (Fig. 9). As an input we used all DEG ($P < 0.01$, FDR < 0.1, log2-Ratio > 0.5 for upregulated genes, or < −0.5 for downregulated genes) from each contrast. Expression of 83 genes (regardless of log2-Ratio) was altered in both contrasts. Interestingly, all overlapped genes were regulated in the same manner in each comparison, i.e. 48 genes were up- (log2Ratio > 0.5) and 35 genes were down-regulated (log2-Ratio < −0.5) in the prepartum luteolysis and antigestagen-induced luteolysis groups compared to mid-gestation. These genes were associated with functional terms related to cellular death, vascular lesions and migration of immune cells. The complete list of genes from each intersection as well as enriched functional terms by commonly regulated genes with statistical details is provided in Supplemental File 5.

3.6. Expression of candidate target genes

Semi-quantitative real time TaqMan qPCR (RT-qPCR) was applied to evaluate the expression of sixteen (16) selected target genes, which were chosen from DEG from particular contrasts (Fig. 10, Supplemental File 6). Candidates included representative genes from the following functional groups, identified as being affected during either prepartum or/and abortion: *IGF* system (*IGF-1, IGFBP3, IGFBP4, IGFBP5*), *TGF-β*

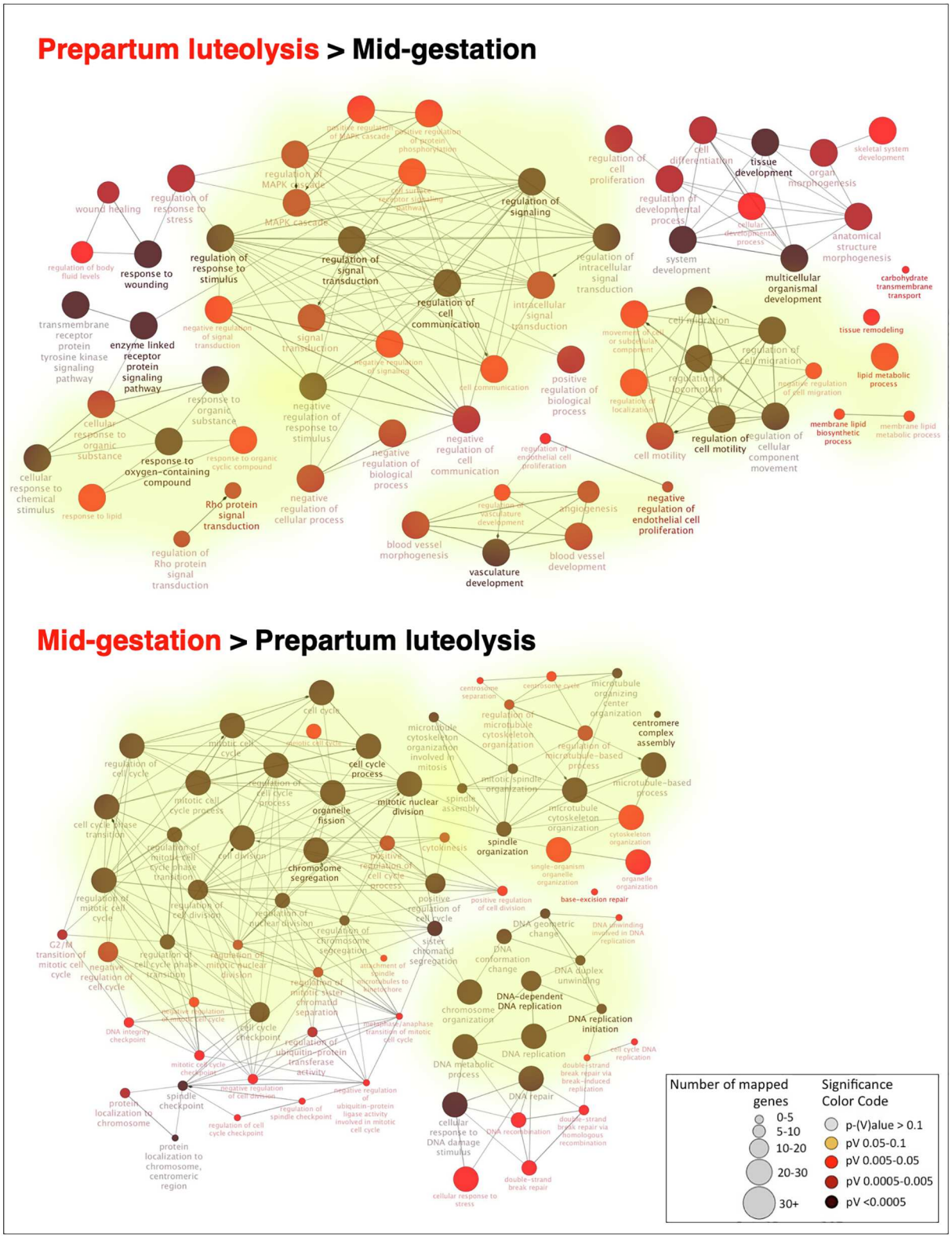

Fig. 4. Functional networks of the contrast "prepartum luteolysis over mid-gestation" by ClueGO application of Cytoscape. The functional terms overrepresented in each group are shown. Redundant and non-informative terms were removed, and the resulting network was manually rearranged. The number of mapped genes is indicated by the node size, whereas the level of enhancement is denoted by node color (see figure legend). The most represented functional networks during prepartum luteolysis (upper scheme) were associated with: vasculature development, cell motility and migration, lipid metabolic processes, regulation of cell communication, and cellular response to stimulus. Networks more highly represented during mid-gestation (lower scheme) were related to DNA metabolic processes, chromosome segregation, cytoskeleton organization, cell division and cell cycle.

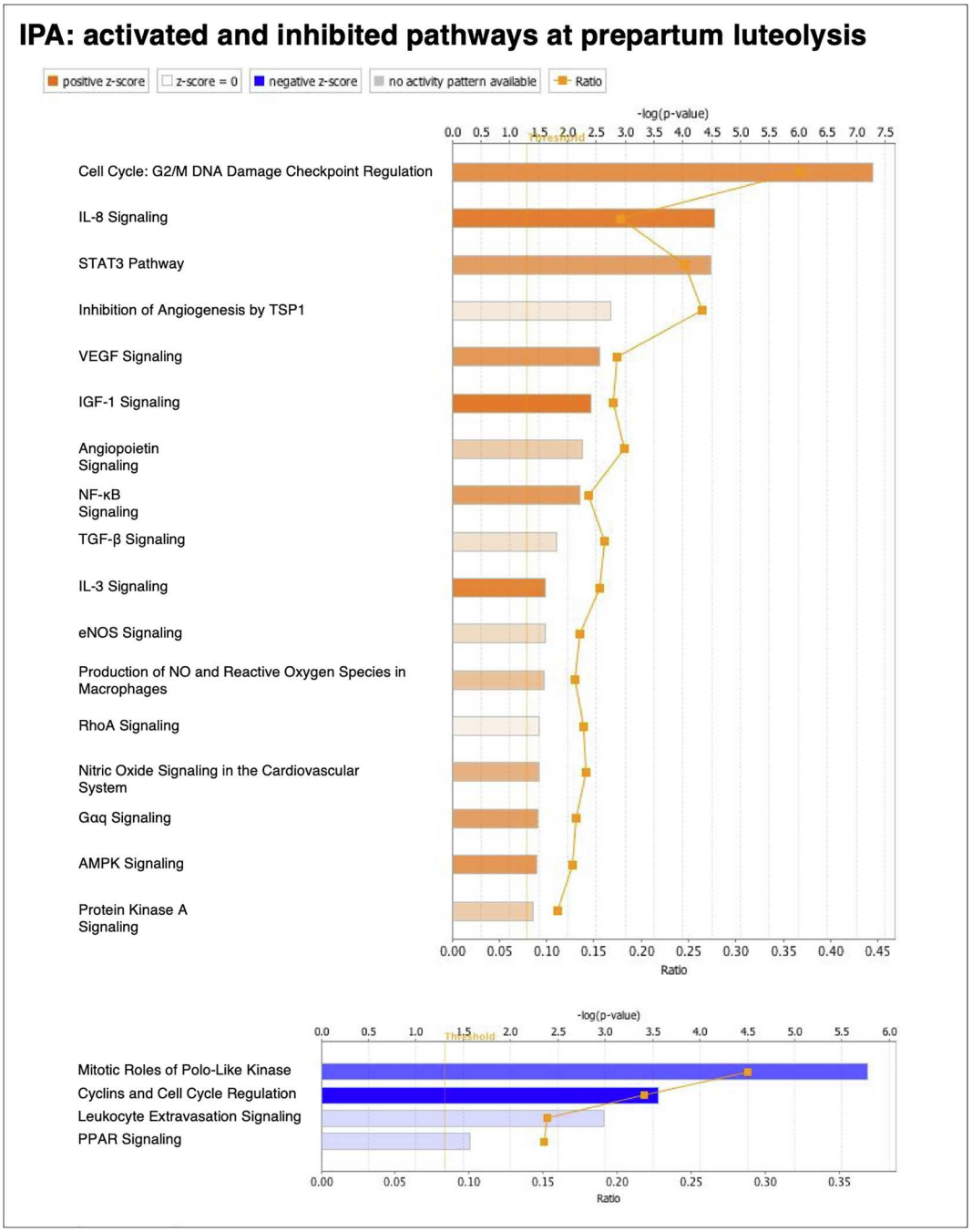

Fig. 5. The canonical pathways enriched for the comparison "prepartum luteolysis over mid-gestation" ranked according to –log (P-value). The color (orange to blue) indicates predicted activity pattern of a given pathway at prepartum luteolysis: blue for inhibition, orange for activation. The overlapping of gene sets is indicated by the orange line (Ratio).

system (*TGFBETA2, TGFBETA3, TGFBR1, TGFBR2*), *IL-8* (*CXCL8*) and its receptor (*CXCR2*) and potential P4 targets (*ICAM1, HSD11B2, ADIPOQ*). Additionally, inhibin subunits beta (*INHBA, INHBB*) and transferrin (*TF*) were included in the group of targets for data validation. Generally, results from RT-qPCR and RNA-Seq were well correlated. Thus, expression of *IGF* system members was upregulated in prepartum luteolysis in comparison to the mid-gestation group (Fig. 10A, Supplemental File 6). Additionally, for the contrast "prepartum luteolysis over mid-gestation", increased expression of the *TGF-β* system was observed, except for *TGFBETA2* (Fig. 10A). The latter as well as *IL-8* (*CXCL2*) did not vary significantly in RT-qPCR between the two experimental groups, although their expression was predicted to be increased by RNA-Seq results. In addition, during prepartum luteolysis, expression of both inhibin subunits beta (*INHBA, INHBB*), *TF, ICAM1* and *ADIPOQ* was also increased (Fig. 10A). Regarding HSD11B2, its expression was downregulated in both prepartum and induced luteolysis when compared to mid-gestation (Fig. 10A and B). Moreover, in the contrast "antigestagen over mid-gestation", a significant increase of *IGFBP5* was found in samples derived from the antigestagen-treated group (Fig. 10B, Supplemental File 6). The expression of *TF*, however, did not differ, although it was predicted to be upregulated in the RNA-Seq dataset. Comparison of induced luteolysis and prepartum luteolysis groups revealed higher expression of almost all investigated target genes (i.e., *IGF1, IGFBP3, IGFBP4, INHBA, INHBB, TGFBETA2, TGFBETA3, TGFBR2, ADIPOQ*) during natural prepartum (Fig. 10C, Supplemental File 6). On the other hand, *HSD11B2* was more highly

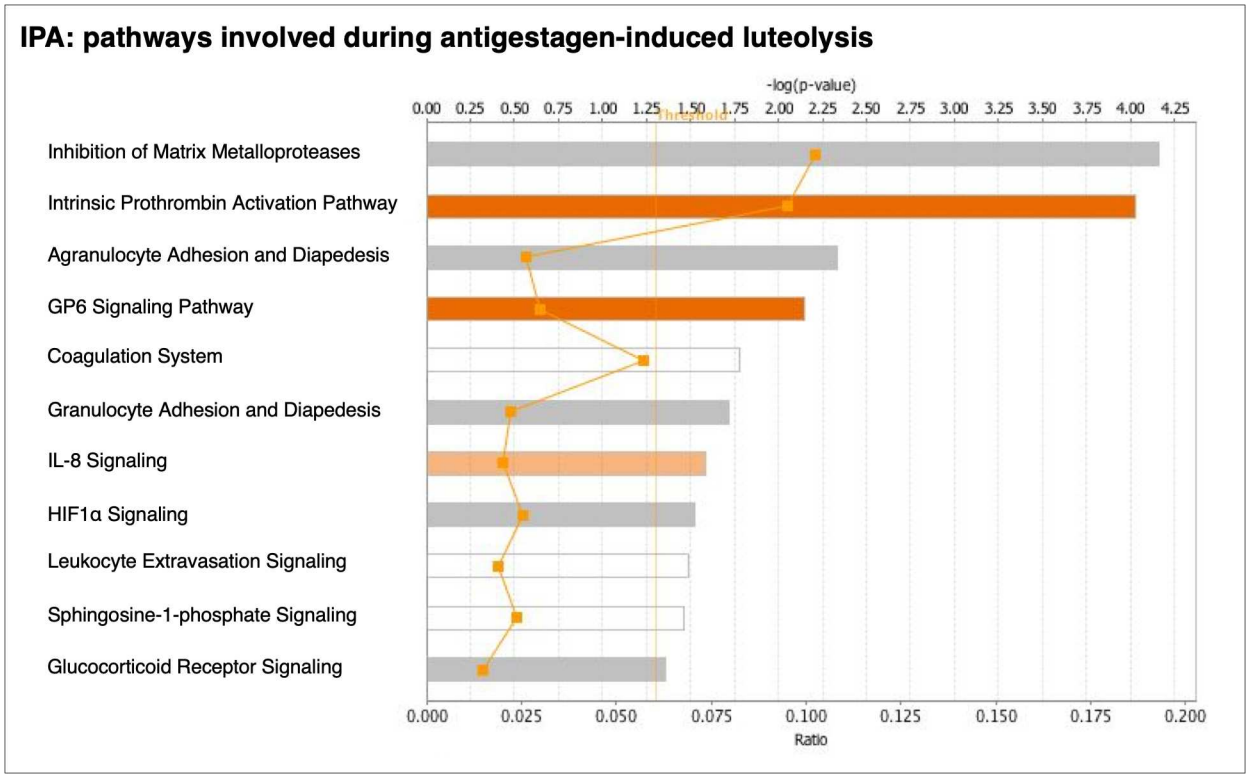

Fig. 6. The canonical pathways enriched for the comparison "antigestagen over mid-gestation" ranked according to –log (P-value). The color indicates predicted activity pattern of a given pathway in the induced luteolysis group: whereas orange indicates activation; for white and grey, although enriched, no activation status could be predicted from the software (no activity pattern available). The overlapping of gene sets is indicated by the orange line (Ratio).

expressed during induced luteolysis (Fig. 10C). Finally, expression of *CXCR2*, i.e., IL-8 receptor, did not significantly differ between these two conditions, but its expression was predicted to be higher in the induced luteolysis group.

4. Discussion

The cascade initiating parturition in the dog seems to be regulated at the level of the nuclear P4 receptor localized in the maternal part of the placenta (Kowalewski et al., 2010). Despite individual, diurnal and breed-associated fluctuations in peripheral P4 concentrations, labor is observed in bitches at approximately 63 +/− 2 days after mating. At that time, circulating P4 levels typically drop below 2–3 ng/ml (Concannon et al., 1989; Hoffmann et al., 1994; Verstegen-Onclin and Verstegen, 2008). A simultaneous increase in PGF2α, most probably acting as both a luteolytic and a uterotonic factor, is observed (Nohr et al., 1993). However, the molecular regulation of the cascade initiating parturition in the dog and its involvement in the underlying feto-maternal communication has not yet been completely elucidated. In particular, the complex P4-mediated effects remain to be better understood. Therefore, here, by applying next generation sequencing techniques, we investigated global transcriptome changes in the canine placenta. Mid-term placentae were compared with normal and antigestagen-induced luteolysis. A further, clinically important, comparison was made between the two luteolytic groups, to reveal molecular mechanisms underlying physiological parturition and abortion.

4.1. Prepartum luteolysis

When compared with mid-gestation, the prepartum placenta was characterized by the strong presence of functional terms associated with active apoptosis as well as impaired cellular adhesion and proliferation, with particular respect to endothelial cells. This was also reflected in the overall classification of identified genes compared with the reference genome. This pointed towards a functional shift from proliferation-associated processes prevailing during mid-gestation to increased cell communication and suppressed proliferative activities during the prepartum luteolysis. The observed changes were further mirrored in overrepresented functional networks and in a perturbed balance between two angiogenic factors, *ANGPT2* and *VEGFA*. Thus, whereas *ANGPT2* was strongly upregulated, the expression of *VEGFA* was decreased in our dataset during prepartum luteolysis. In regard to *VEGFA*, our RNA-Seq findings corroborate the previously observed decreased expression of *VEGFA* in canine utero-placental units at prepartum (Gram et al., 2015a). A functional interchange between ANGPT2 and VEGFA has been shown before (Maisonpierre et al., 1997). Accordingly, in the presence of VEGFA, ANGPT2 serves as an angiogenic signal, promoting endothelial cell migration and proliferation. However, in the absence of VEGFA, ANGPT2 causes loosening of cell-matrix adhesion, thereby contributing to endothelial cell apoptosis and consequently to vascular degeneration (Hanahan, 1997; Maisonpierre et al., 1997). Whether this mechanism applies to the canine placenta and contributes to vascular disintegration and, possibly, to the post-partum detachment of the placenta in the dog, needs to be further clarified. Interestingly, however, following this idea, we identified *ICAM1*, an intercellular adhesion molecule, among the factors more highly represented at prepartum luteolysis. Its role is associated with loosening of the barrier function of endothelial cells (Yang et al., 2005; Anbarasan et al., 2015). Under normal, physiological conditions, *ICAM1* is stable and expressed at a relatively low level in endothelial cells (Anbarasan et al., 2015). However, in response to inflammatory mediators such as TNF, the expression of *ICAM1* dramatically increases, promoting the attachment of leukocytes to endothelial cells, recruiting them to the sites of inflammation and thereby facilitating migration of leukocytes (Yang et al., 2005; Anbarasan et al., 2015). Besides loosening cell-to-cell contacts in endothelial cells, ICAM1 also stimulates local production of pro-inflammatory cytokines and

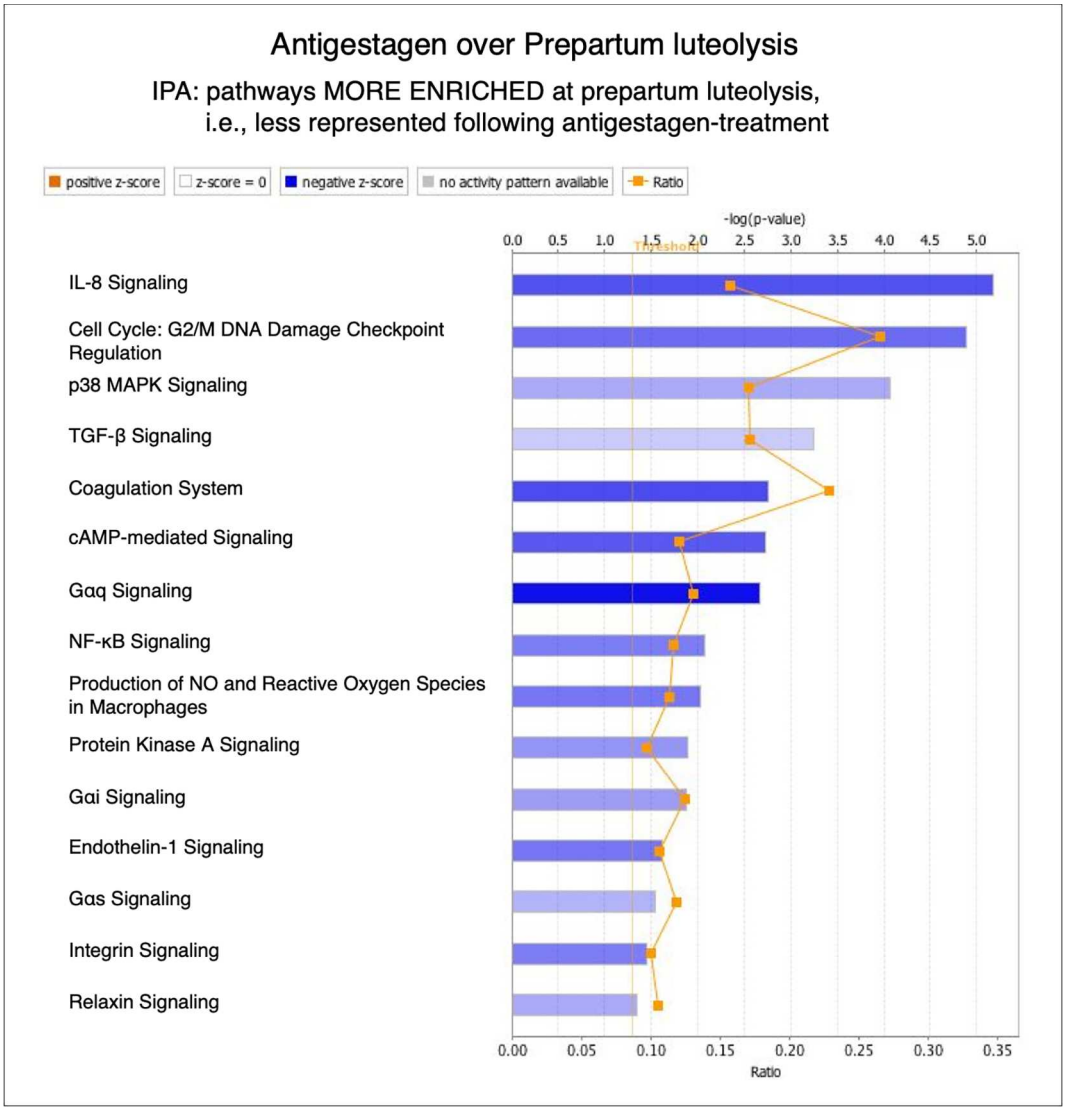

Fig. 7. The canonical pathways enriched for the comparison "antigestagen over prepartum luteolysis" ranked according to –log (P-value). The blue color indicates predicted inhibition of a given pathway at antigestagen-induced luteolysis (in reference to normal prepartum luteolysis). The overlapping of gene sets is indicated by orange line (Ratio).

chemokines, additionally attracting immune cells to inflamed areas (Yang et al., 2005; Ramos et al., 2014). In this regard, pathways recognized to be activated at prepartum, like IL-8-, IL-3-, eNOS-, RhoA- and NO-signaling, further imply essential involvement of mechanisms regulating disruption of placental vascular function during the onset of canine labor. It is noteworthy that these processes seem to occur in parallel, or as a result of local modulation of immune system directed towards inflammation. This was further emphasized in our dataset by strong enrichment of proinflammatory pathways, such as NF-κB-, AMPK- and TGF-β-mediated signaling. TGF-β is a multifunctional cytokine, whose effects are tissue and/or condition-specific, but generally include anti-proliferative, anti-angiogenic and pro-apoptotic actions (Jones et al., 2006; Kubiczkova et al., 2012; Massague, 2012). The involvement of TGF-β-mediated signaling in active luteolysis was addressed in cattle (Maroni and Davis, 2011, 2012) and discussed for the canine species (Zatta et al., 2017). Additionally, a possible role of TGF-β in fetal membrane detachment was proposed for cattle (Hirayama et al., 2015). In the data set presented herein, increased placental expression of several members of the *TGF-β*-system, i.e., *TGFBETA3, TGFBR1* and *TGFBR2*, was confirmed by RT-qPCR during prepartum. In view of the above-mentioned facts and our own observations, the TGF-β-family comes across as being important molecules in the dog. Further mechanistic studies are required to determine the role of TGF-β in the utero-placental signaling cascade initiating canine labor.

It needs to be emphasized that functional changes implied from the comparison of transcriptomes between prepartum luteolysis, representing late placenta, and mid-term placenta are certainly additionally influenced by the continuous exposure to gradually decreasing P4 levels during the second half of gestation and are thereby time-related. These time-related processes could be referred to as the process of placental maturation towards preparation for parturition. In a broader sense, this could also provide, at least in part, an explanation for lower PGF2α levels induced in the placenta during the pre-term withdrawal of P4, compared with natural luteolysis (Baan et al., 2008; Kowalewski et al., 2010). The process of placental maturation and aging of the placenta is also seen in the functional terms more strongly represented during mid-gestation and relating to increased proliferative activity, e.g., initiation of DNA replication or positive regulation of cell division and chromosome segregation, which all cease in the late term placenta.

We have also validated the expression of two subunits of inhibin beta: inhibin beta A (*INHBA*) and inhibin beta B (*INHBB*), which are

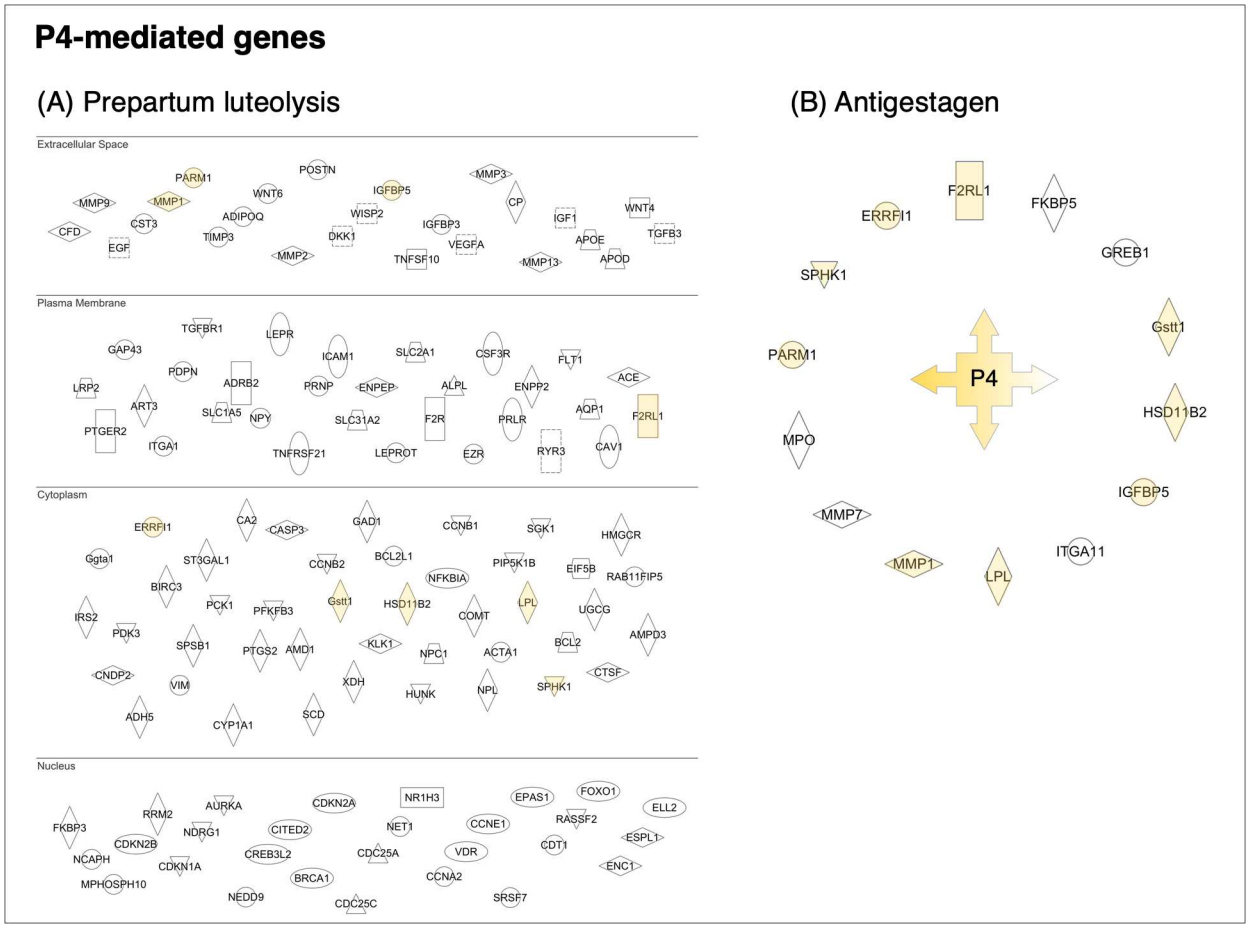

Fig. 8. Genes potentially regulated by progesterone (P4) as determined by Ingenuity Pathway Analysis (IPA®, Qiagen) in (A) prepartum luteolysis and (B) induced luteolysis group. Highlighted in orange are genes found to be common for both groups. For abbreviations see Supplemental File 7.

members of the family of inhibin-related proteins. Their expression was significantly increased during prepartum and was more strongly represented at normal than during induced parturition. This group of proteins belongs structurally to the TGF-β family, and includes activins and inhibins (Wijayarathna and de Kretser, 2016). It needs to be clarified that structural differences, and consequently opposing functional properties between activins and inhibins, exist in their subunit compositions. Whereas activins are homo- or hetero-dimers composed of two beta subunits (INHBA or INHBB), and thus are called activin A, AB or B, respectively, inhibins are heterodimers of subunit alpha (INHA) and any one of the beta subunits (Wijayarathna and de Kretser, 2016). In the present study, the expression of subunit alpha was not verified by RT-qPCR, as it did not vary in any RNA-seq dataset. On the other hand, increased expression of *INHBA* as well as *INHBB* was found in the prepartum luteolysis group. This finding, together with unchanged expression of subunit alpha, implies preferential synthesis in favor of activins. Similarly, in women, serum activin A peaks at 38–39 weeks of pregnancy (O'Connor et al., 1999; Wijayarathna and de Kretser, 2016), and rapidly drops following delivery, indicating its role at the end of gestation. Activins appear to act as pro-inflammatory and proapoptotic agents (Jones et al., 2006; Wijayarathna and de Kretser, 2016), and are known to affect functions of endothelial cells, which show signs of oxidative stress when exposed to activins (Yu et al., 2012). Cumulatively, in the canine placenta activins could be involved in prepartum-associated modulation of immune and vascular functions and therefore merit more attention in future.

As already mentioned, among significantly enriched functional terms and networks during prepartum luteolysis were those related to intracellular signal transduction and cell communication, indicating intensified cellular cross-talk in the placenta prior to delivery. Moreover, enhanced cellular communication was reflected in activated pathways, like IGF1 signaling, G-protein-coupled receptor signaling or PKA pathway. Insulin-like growth factors (IGFs) are growth-promoting peptides which stimulate cell proliferation and differentiation (Jones and Clemmons, 1995). Their bioavailability and signaling is dependent on the presence of IGF binding proteins (IGFBPs), which by interfering with IGFs may affect their half-life and affinity for receptors (Jones and Clemmons, 1995; Allard and Duan, 2018). The majority of IGF effects have been described predominantly for early stages of pregnancy. Thus, during human gestation, they were shown to be important for placentation and fetal development (Fowden, 2003; Hiden et al., 2009; Lauszus and Fuglsang, 2016). Their role has been addressed also for the dog, in which IGFs serve as markers of decidualization (Kautz et al., 2014). On the other hand, only sparse information is available about expression of *IGFs*, their signaling and possible actions prior to parturition. In the dataset presented herein, we have observed, and confirmed by RT-qPCR, increased availability of transcripts encoding for placental *IGF1* and several *IGFBPs* in the prepartum luteolysis group. Similarly, in humans, serum maternal IGF1 gradually increases throughout pregnancy, reaching its highest levels around the 37th week of gestation (Wiesli et al., 2006). The potential role of these increased levels of IGF1 during human gestation is considered to be related to the secretion of placental growth hormone (GH) (Caufriez et al., 1990). Regrettably, nothing is known about synthesis and secretion of GH in the canine placenta, and our data are the first to report increased placental *IGF1* expression at term in this species. Therefore, a potential relationship between GH and IGF1 during late stages of canine gestation, as described for humans, remains unclear and certainly raises a topic worth further

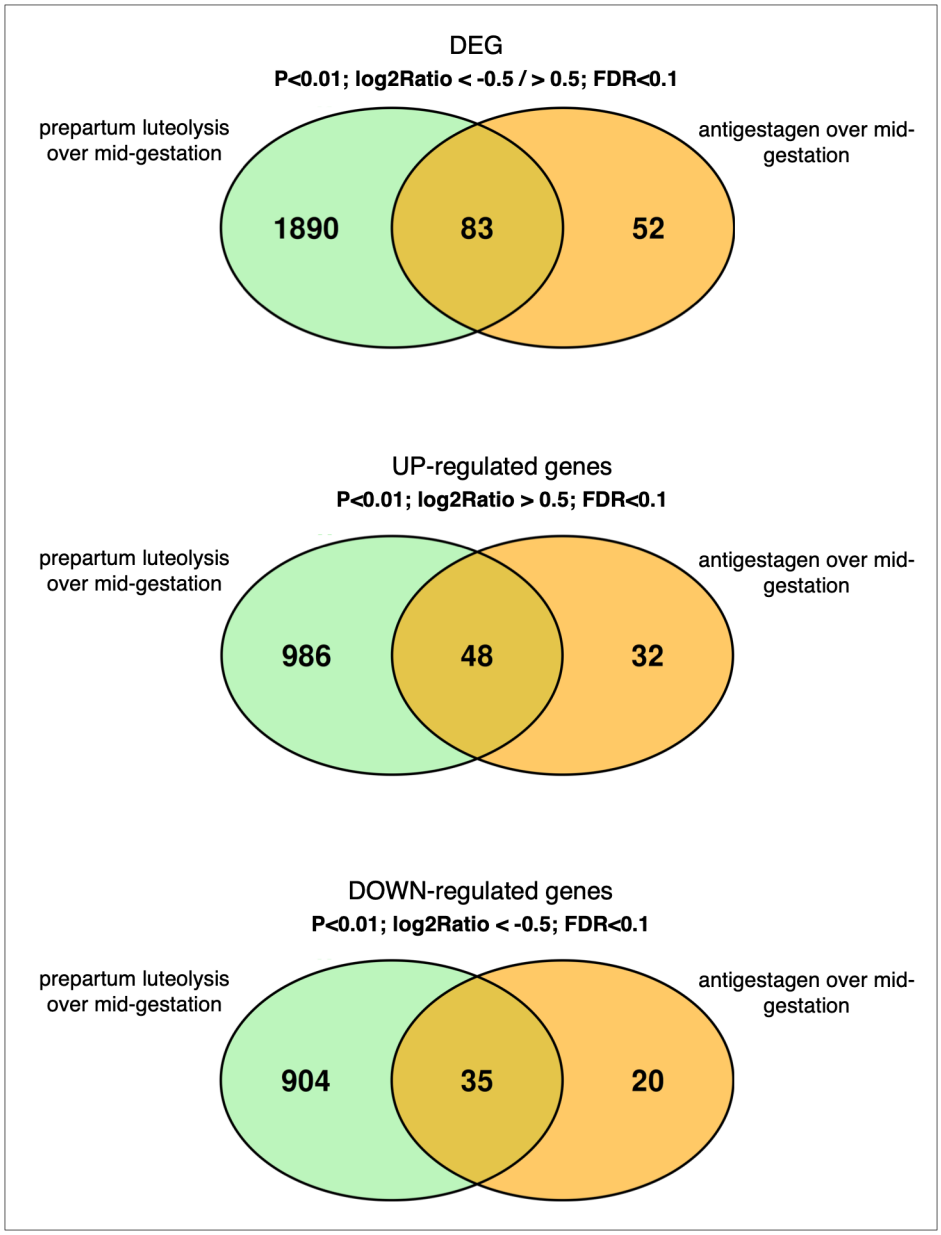

Fig. 9. Venn diagrams showing the distribution and overlap of DEG between the contrasts "prepartum luteolysis over mid-gestation" and "antigestagen over mid-gestation". As an input, lists of DEG ($P < 0.01$, $FDR < 0.1$) from respective comparisons were used. Presented are overlaps of: all DEG (log2Ratio > 0.5 or < -0.5), upregulated genes (log2Ratio > 0.5) and downregulated genes (log2Ratio < -0.5). When full sets of genes were used, 83 genes were found in both contrasts. They revealed the same direction of regulation in each comparison, i.e.: 48 genes were up- and 35 genes were down-regulated in prepartum luteolysis and antigestagen-induced luteolysis groups compared with mid-gestation. The complete list of genes from each intersection is provided in Supplemental File 5.

investigation. The possible endocrine functions of IGF1 in the maternal circulation, e.g., in supporting development of the mammary gland, should be taken into consideration (Duclos et al., 1989; Macias and Hinck, 2012; Bao et al., 2016).

Remarkably, among the overrepresented functional networks linked to prepartum luteolysis we identified terms and genes related to positive metabolism of lipids, particularly to cholesterol. In this context, even more interesting was the detection of decreased levels of *HSD11B2*. Its lower expression at prepartum was confirmed by qPCR. HSD11B2 is responsible for conversion of cortisol to its inactive form, cortisone (Ulick et al., 1977; Funder et al., 1988). This observation has drawn our attention to the potential involvement of cortisol in canine parturition. Admittedly, the role of placental HSD11B2 has been addressed in other species, like sheep, humans and rats (Murphy et al., 1974; Sun et al., 1999; Clarke et al., 2002; Mark et al., 2009), and its increased expression has been considered to be protective in preventing overexposure of the fetus and placenta to high maternal cortisol levels secreted, for example, in response to stress (Sun et al., 1998; Burton and Waddell, 1999). The decreased expression of *HSD11B2* observed herein somewhat resembles the situation reported in the prepartum sheep placenta, in which *HSD11B2* was suppressed (Clarke et al., 2002). In the ovine species, however, the low prepartum *HSD11B2* was predominantly attributed to enhancement of fetal cortisol facilitating placental estrogen steroidogenesis (Mason et al., 1989). Such a mechanism is clearly precluded in dogs because they lack placental steroidogenesis (Hoffmann et al., 1994; Nishiyama et al., 1999). Nevertheless, as mentioned elsewhere, the function of cortisol during the parturition cascade in dogs has been discussed before (Concannon et al., 1978; Hoffmann et al., 1994; Veronesi et al., 2002). In fact, in some studies, glucocorticoids were used to terminate gestation in the dog (Austad et al., 1976; Zone et al., 1995; Wanke et al., 1997). However, since high dosages and repeated applications were required, whether this reflects the normal prepartum cascade is questionable. The possible action of glucocorticoids at prepartum in the dog should be considered in the context of availability of their receptor (GR/NR3C1), as it might be important for the functional withdrawal of P4 at the placental level (Gram et al., 2016). Accordingly, although placental expression of *GR/NR3C1* is elevated at normal prepartum luteolysis, it does not appear to be crucial for induction of the prostaglandin cascade (Gram et al., 2016). The role of cortisol in regulating the expression and

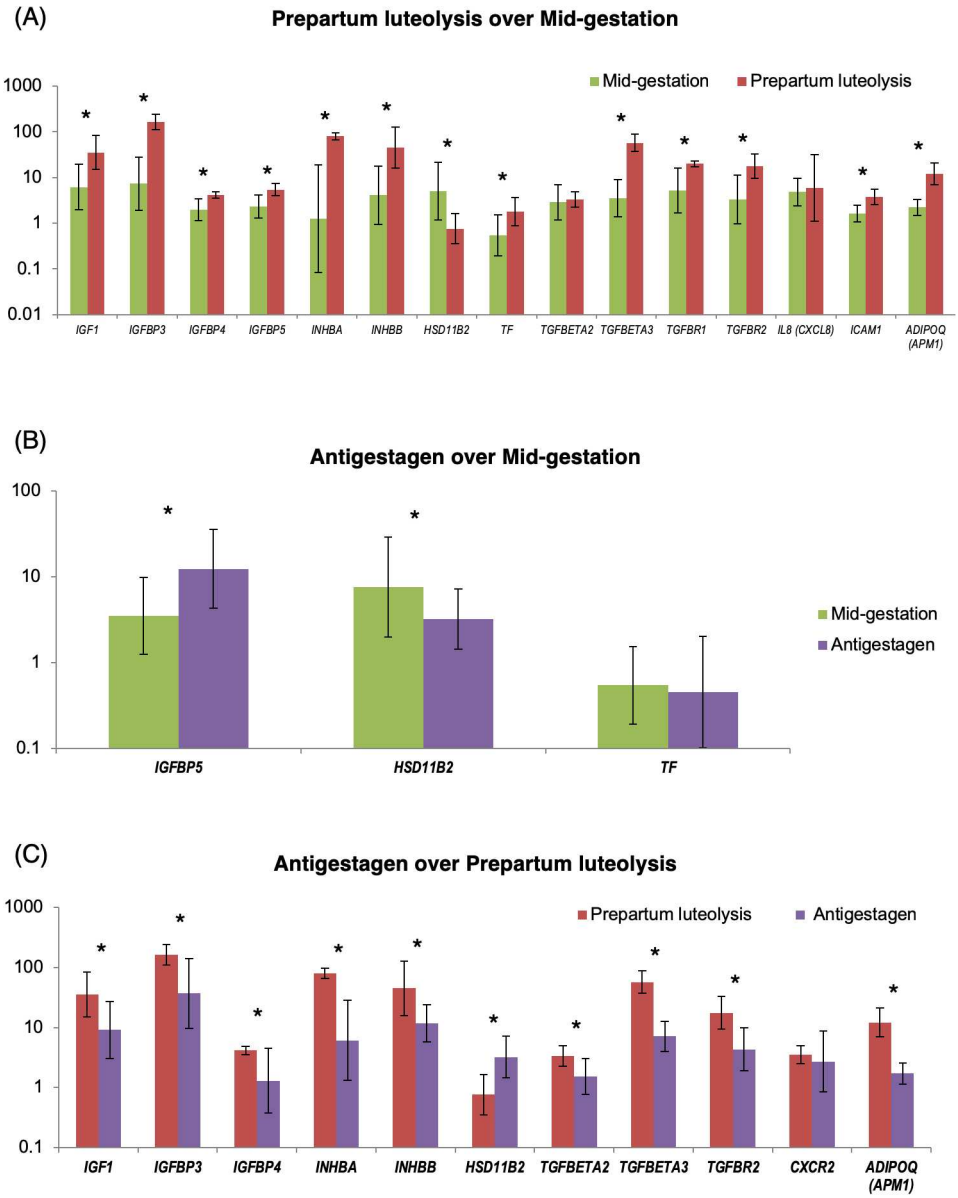

Fig. 10. The relative gene expression (RGE) of candidate genes as determined by real-time TaqMan qPCR (RT-qPCR). Numerical data are presented on a logarithmic scale as geometric means Xg ± geometric standard deviation (SD). An unpaired, two-tailed Student's *t*-test was applied. An asterisk indicates statistical significance between the particular groups ($P < 0.05$). See Table 1 for abbreviations and Supplemental File 6 for statistical details.

function of its own receptor in the canine placenta, although not yet described, should also be considered. Finally, although the overall systemic concentrations of cortisol in parturient bitches vary strongly individually (Concannon et al., 1978; Hoffmann et al., 1994; Veronesi et al., 2002), nothing is known about its local levels in the placenta, or about the contribution of the fetal HPA axis to cortisol production. Therefore, in view of these facts and our current results, it appears plausible that HSD11B2 could be involved in enhancing local availability of cortisol prior to term, but the consequences of this event still need to be explored.

4.2. Antigestagen-induced changes

As evidenced from some previous studies, interfering with PGR function by treatment with the antigestagen aglepristone results in the cessation of luteal function, a rise in peripheral PGF2α and abortion. In order to better understand mechanisms underlying the antigestagen-induced termination of canine pregnancy, we compared placental transcriptomes of aglepristone-treated mid-term bitches with non-treated controls at the same stage of pregnancy. In this context, it is of fundamental importance to understand that the end result of aglepristone treatment, i.e., abortion, results from the reciprocal placental feto-maternal communication (see review in (Kowalewski et al., 2015)). By establishing this contrast, we shed light on the role of functional withdrawal of P4 at the level of its nuclear receptor PGR.

As revealed by downstream analysis of 135 DEG, the changes in placental transcriptome induced by antigestagen treatment to a great extent matched those changes observed during natural prepartum. This was reflected in overall functional representation of genes in reference to the whole canine genome, clearly indicating a functional shift towards the cell communication sub-cluster, with fewer genes being linked to cell cycle and proliferation sub-groups. Also, functional terms and pathways referred predominantly to alterations in placental vascularity and activation of the immune system. These observations are also consistent with a study describing morphological changes in the placenta in dogs after aglepristone treatment (Steiger et al., 2006).

These changes included degeneration of the placental labyrinth, endothelial swelling and detachment of the trophoblast, and correspond with the increased hypoxia and endothelin signaling found in the present study. The potential involvement of endothelins in initiating the signaling cascade of prostaglandin synthesis during normal and induced parturition in the dog was also implied in some previous studies (Gram et al., 2015b, 2017).

4.3. Normal vs. induced luteolysis and P4-mediated genes

In our next step, common changes induced in placental transcriptomes during normal and induced luteolysis in reference to mid-gestation were visualized using Venn diagrams. Notably, all of the 83 commonly expressed genes were regulated in the same manner, i.e., were either upregulated or downregulated under both conditions, and were associated predominantly with cellular death, vascular lesion and cellular infiltration by immune cells.

They also shared similar upstream regulators, such as TNF, TGF-β, IGF1, PPARG and dexamethasone. This even more strongly highlights the importance of P4/PGR, and of maternal decidual cells, in controlling the maintenance and termination of pregnancy in the dog. In this context, being expressed solely in the fetal trophoblast throughout canine gestation, the functional involvement of PPARG in regulating canine placental function was suggested from a previous study (Kowalewski et al., 2011). PPARG is indeed an interesting candidate gene as it acts as an alternative receptor for arachidonic acid-derived metabolites, such as prostaglandins (Berger and Moller, 2002). Its withdrawal during normal and antigestagen-induced luteolysis may play a functional role during the prepartum endocrine cascade in the dog, leading to detachment of the placenta (Kowalewski et al., 2011). Therefore, in the next step, our focus was on the identification of P4-mediated genes. By applying IPA® software, the majority of the genes found in the contrast "induced luteolysis over mid-gestation" was also observed in the natural prepartum luteolysis group, further emphasizing the similarity of molecular pathways activated in both conditions, and supporting our previously postulated hypothesis of the importance of P4 withdrawal for initiation of the luteal cascade in the dog (Kowalewski et al., 2010). Among representative P4-mediated genes revealing similar expression patterns under both conditions were: *HSD11B2* and *IGFBP5* (one of the IGFs binding proteins, (Schneider et al., 2002)), *F2RL1* (mediator of immune response, involved in stimulation of IL-8 release (Asokananthan et al., 2002)) and *MMP1* (involved in extracellular matrix remodeling, implicated in the rupture of fetal membranes at labor in women (Maymon et al., 2000)). In view of these observations, P4, acting through PGR, appears to be among the key molecules determining placental integrity in the dog. Moreover, based on our findings in the abortion group, P4 functions include vascular maintenance, support of proliferation of cells, and immune system-related effects pointing towards immunosuppression.

4.4. Similar but not the same: defining molecular differences of clinical importance between natural parturition and abortion induced at mid-term

Although, as indicated above, the signatures of molecular changes occurring at antigestagen-induced abortion appeared similar to those observed during normal prepartum, however, clinical observations indicate important differences between these two processes (Hoffmann et al., 1999; Fieni et al., 2001). Firstly, the increase of PGF2α in the maternal circulation of antigestagen-treated dogs, although sufficient for luteolysis, does not closely reflect the concentrations observed during normal prepartum (Nohr et al., 1993; Hoffmann et al., 1999; Baan et al., 2008). This was also reflected in our dataset showing higher representation of prostaglandin-related pathways by placing *PTGS2/COX2* among the top P4-regulated genes during normal but not during induced luteolysis. Consequently, at the clinical level, uterine contractions appear weaker and the passage of fetuses is hindered (Galac et al., 2000; Pettersson and Tidholm, 2009; Rigau et al., 2011). This, in turn, results in extended duration of abortion. Moreover, in contrast with normal prepartum luteolysis, there is no utero-placental increase in glucocorticoid receptors (*GR/NR3C1*) following antigestagen treatment (Gram et al., 2016). The underlying molecular mechanisms of parturition and abortion were investigated previously, but predominantly in the context of normally developing pregnancy, and have not been compared between each other. Therefore, aiming to provide better understanding of the physiology of canine labor and to determine differences between parturition and abortion at the molecular level that could have been overlooked in previous studies, we compared placental transcriptomes between antigestagen-induced luteolysis and prepartum luteolysis. We found a high number (1509) of DEG between these two groups. Generally, genes more highly expressed during natural prepartum were associated with similar functional terms as observed for prepartum luteolysis when compared to mid-gestation. This included negative regulation of angiogenesis, immune system related processes and cytokine production. Similarly, most of the identified pathways (e.g., IL8-, TGF-β-, NF-κB-, or PKA-signaling) were in accordance with those observed for the pairwise comparison "prepartum luteolysis over mid-gestation", and showed higher enrichment and activation for the prepartum than induced luteolysis groups. This was also supported by higher expression of candidate genes found by qPCR, indicating, e.g., higher expression of the *TGF-β* system. Thus, although sharing similar P4-dependent pathways important for initializing the luteolytic cascade, both induced and normal parturition appear to differ at the level of enhancement of the underlying signaling cascades, leading to differences at the clinical level. These differences could relate to the aforementioned maturation of the canine placenta towards prepartum. Continuous exposure to decreasing P4 levels and ageing processes could play roles in the underlying mechanisms.

5. Conclusions

Here, for the first time the RNA-seq approach has been applied to investigate global transcriptome changes in the canine placenta associated with the initiation of parturition. The data presented herein contribute to better understanding of the prepartum cascade initialized in the placenta, in particular with regard to underlying cell-to-cell cross talk between the maternal and fetal compartments. Events occurring in the placenta at term appear to be predominantly associated with apoptosis, immune system activation and disturbance of vasculature. Ultimately, besides inducing prepartum PGF2α release, these processes lead to detachment of the placenta and expulsion of fetuses.

By studying transcriptomic changes induced by antigestagen treatment, potential P4-mediated genes and effects could be distinguished and referred to pre-parturient events. Moreover, several hypotheses were put forward and factors not previously considered were identified, providing a basis for further investigations of the molecular and endocrine cascade terminating canine gestation. However, because our study involved a rather low number of animals, our findings are not definitive, and further studies with larger numbers of animals are required to support and verify our hypotheses. Nevertheless, some of the factors, like activins, the IGF and TGF systems or locally acting cortisol, certainly deserve closer attention and should determine future research directions.

The similarity of the identified changes between natural and aglepristone-induced luteolysis further highlights the importance of P4 and of PGR-expressing decidual cells at the onset of luteolysis.

From the clinical point of view the difference in levels of activation of molecular pathways and functional networks between normal and induced luteolysis needs to be taken into account. It indicates an important role for temporal exposure of the placenta to gradually decreasing P4, which needs to be examined further in order to be translated into clinical relevance.

Funding

This work was supported by The Swiss National Science Foundation (SNSF) research Grant Number 31003A_182481 to MPK.

Acknowledgements

Authors are grateful to Dr. Barry Bavister for careful editing of the manuscript and to Prof. Dr. Selim Aslan, Near East University, Cyprus (formerly of the University of Ankara, Turkey) and to Prof. Dr. Bernd Hoffmann, Justus-Liebig University Giessen, Germany and their teams for provision of the tissue material. Part of the laboratory work was performed using the logistics at the Center for Clinical Studies, Vetsuisse Faculty, University of Zurich. The technical expertise and contributions of Dr. Jelena Kühn-Georgijevic from Functional Genomic Center ETH/UZH Zurich are gratefully appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.05.019.

References

Allard, J.B., Duan, C., 2018. IGF-binding proteins: why do they exist and why are there so many? Front. Endocrinol. (Lausanne) 9, 117.
Anbarasan, C., Bavanilatha, M., Latchumanadhas, K., Ajit Mullasari, S., 2015. ICAM-1 molecular mechanism and genome wide SNP's association studies. Indian Heart J. 67, 282–287.
Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250.
Anderson, A.B., Flint, A.P., Turnbull, A.C., 1975. Mechanism of action of glucocorticoids in induction of ovine parturition: effect on placental steroid metabolism. J. Endocrinol. 66, 61–70.
Asokananthan, N., Graham, P.T., Fink, J., Knight, D.A., Bakker, A.J., McWilliam, A.S., Thompson, P.J., Stewart, G.A., 2002. Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. J. Immunol. 168, 3577–3585.
Austad, R., Lunde, A., Sjaastad, O.V., 1976. Peripheral plasma levels of oestradiol-17 beta and progesterone in the bitch during the oestrous cycle, in normal pregnancy and after dexamethasone treatment. J. Reprod. Fertil. 46, 129–136.
Baan, M., Taverne, M.A., de Gier, J., Kooistra, H.S., Kindahl, H., Dieleman, S.J., Okkens, A.C., 2008. Hormonal changes in spontaneous and aglepristone-induced parturition in dogs. Theriogenology 69, 399–407.
Bao, Z., Lin, J., Ye, L., Zhang, Q., Chen, J., Yang, Q., Yu, Q., 2016. Modulation of mammary gland development and milk production by growth hormone expression in GH transgenic goats. Front. Physiol. 7, 278.
Berger, J., Moller, D.E., 2002. The mechanisms of action of PPARs. Annu. Rev. Med. 53, 409–435.
Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.H., Pages, F., Trajanoski, Z., Galon, J., 2009. ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25, 1091–1093.
Brown, A.G., Leite, R.S., Strauss 3rd, J.F., 2004. Mechanisms underlying "functional" progesterone withdrawal at parturition. Ann. N. Y. Acad. Sci. 1034, 36–49.
Burton, P.J., Waddell, B.J., 1999. Dual function of 11beta-hydroxysteroid dehydrogenase in placenta: modulating placental glucocorticoid passage and local steroid action. Biol. Reprod. 60, 234–240.
Caufriez, A., Frankenne, F., Englert, Y., Golstein, J., Cantraine, F., Hennen, G., Copinschi, G., 1990. Placental growth hormone as a potential regulator of maternal IGF-I during human pregnancy. Am. J. Physiol. 258, E1014–1019.
Chaim, W., Mazor, M., 1998. The relationship between hormones and human parturition. Arch. Gynecol. Obstet. 262, 43–51.
Challis, J.R.G., Matthews, S.G., Gibb, W., Lye, S.J., 2000. Endocrine and paracrine regulation of birth at term and preterm. Endocr. Rev. 21, 514–550.
Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., Ma'ayan, A., 2013. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinf. 14, 128.
Clarke, K.A., Ward, J.W., Forhead, A.J., Giussani, D.A., Fowden, A.L., 2002. Regulation of 11 beta-hydroxysteroid dehydrogenase type 2 activity in ovine placenta by fetal cortisol. J. Endocrinol. 172, 527–534.
Concannon, P.W., 2011. Reproductive cycles of the domestic bitch. Anim. Reprod. Sci. 124, 200–210.
Concannon, P.W., Butler, W.R., Hansel, W., Knight, P.J., Hamilton, J.M., 1978. Parturition and lactation in the bitch: serum progesterone, cortisol and prolactin. Biol. Reprod. 19, 1113–1118.
Concannon, P.W., McCann, J.P., Temple, M., 1989. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. J. Reprod. Fertil. Suppl. 39, 3–25.
Concannon, P.W., Powers, M.E., Holder, W., Hansel, W., 1977. Pregnancy and parturition in the bitch. Biol. Reprod. 16, 517–526.
Csapo, A.I., Pinto-Dantas, C.A., 1965. The effect of progesterone on the human uterus. Proc. Natl. Acad. Sci. U.S.A. 54, 1069–1076.
De Rensis, F., Saleri, R., Tummaruk, P., Techakumphu, M., Kirkwood, R.N., 2012. Prostaglandin F2alpha and control of reproduction in female swine: a review. Theriogenology 77, 1–11.
Duclos, M., Houdebine, L.M., Djiane, J., 1989. Comparison of insulin-like growth factor 1 and insulin effects on prolactin-induced lactogenesis in the rabbit mammary gland in vitro. Mol. Cell. Endocrinol. 65, 129–134.
Fieni, F., Marnet, P.G., Martal, J., Siliart, B., Touzeau, N., Bruyas, J.F., Tainturier, D., 2001. Comparison of two protocols with a progesterone antagonist aglepristone (RU534) to induce parturition in bitches. J. Reprod. Fertil. Suppl. 57, 237–242.
Fowden, A.L., 2003. The insulin-like growth factors and feto-placental growth. Placenta 24, 803–812.
Funder, J.W., Pearce, P.T., Smith, R., Smith, A.I., 1988. Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science 242, 583–585.
Galac, S., Kooistra, H.S., Butinar, J., Bevers, M.M., Dieleman, S.J., Voorhout, G., Okkens, A.C., 2000. Termination of mid-gestation pregnancy in bitches with aglepristone, a progesterone receptor antagonist. Theriogenology 53, 941–950.
Gram, A., Boos, A., Kowalewski, M.P., 2017. Cellular localization, expression and functional implications of the utero-placental endothelin system during maintenance and termination of canine gestation. J. Reprod. Dev. 63, 235–245.
Gram, A., Buchler, U., Boos, A., Hoffmann, B., Kowalewski, M.P., 2013. Biosynthesis and degradation of canine placental prostaglandins: prepartum changes in expression and function of prostaglandin F2alpha-synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD). Biol. Reprod. 89, 2.
Gram, A., Fox, B., Buchler, U., Boos, A., Hoffmann, B., Kowalewski, M.P., 2014. Canine placental prostaglandin E2 synthase: expression, localization, and biological functions in providing substrates for prepartum PGF2alpha synthesis. Biol. Reprod. 91, 154.
Gram, A., Hoffmann, B., Boos, A., Kowalewski, M.P., 2015a. Expression and localization of vascular endothelial growth factor A (VEGFA) and its two receptors (VEGFR1/FLT1 and VEGFR2/FLK1/KDR) in the canine corpus luteum and utero-placental compartments during pregnancy and at normal and induced parturition. Gen. Comp. Endocrinol. 223, 54–65.
Gram, A., Latter, S., Boos, A., Hoffmann, B., Kowalewski, M.P., 2015b. Expression and functional implications of luteal endothelins in pregnant and non-pregnant dogs. Reproduction 150, 405–415.
Gram, A., Trachsel, A., Boos, A., Kowalewski, M.P., 2016. Elevated utero/placental GR/NR3C1 is not required for the induction of parturition in the dog. Reproduction 152, 303–311.
Greven, H., Kowalewski, M.P., Hoffmann, B., Geyer, J., Rex-Haffner, M., Ugele, B., Schuler, G., 2007. Bovine placental steroid sulphatase: molecular cloning and expression pattern in placentomes during gestation and at parturition. Placenta 28, 889–897.
Hanahan, D., 1997. Signaling vascular morphogenesis and maintenance. Science 277, 48–50.
Hatakeyama, M., Opitz, L., Russo, G., Qi, W., Schlapbach, R., Rehrauer, H., 2016. SUSHI: an exquisite recipe for fully documented, reproducible and reusable NGS data analysis. BMC Bioinf. 17, 228.
Heap, R.B., Deanesly, R., 1966. Progesterone in systemic blood and placentae of intact and ovariectomized pregnant guinea-pigs. J. Endocrinol. 34, 417–423.
Hiden, U., Glitzner, E., Hartmann, M., Desoye, G., 2009. Insulin and the IGF system in the human placenta of normal and diabetic pregnancies. J. Anat. 215, 60–68.
Hirayama, H., Koyama, K., Sawai, K., Fujii, T., Naito, A., Fukuda, S., Kageyama, S., 2015. Localization of TGF-beta and TGF-beta receptor in bovine term placentome and expression differences between spontaneous and induced parturition. Placenta 36, 1239–1245.
Hoffmann, B., Hoveler, R., Nohr, B., Hasan, S.H., 1994. Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens. Exp. Clin. Endocrinol. 102, 185–189.
Hoffmann, B., Kyrein, H.J., Ender, M.L., 1973. An efficient procedure for the determination of progesterone by radioimmunoassay applied to bovine peripheral plasma. Horm. Res. 4, 302–310.
Hoffmann, B., Riesenbeck, A., Schams, D., Steinetz, B., 1999. Aspects on hormonal control of normal and induced parturition in the dog. Reprod. Domest. Anim. 34, 219–226.
Iliodromiti, Z., Antonakopoulos, N., Sifakis, S., Tsikouras, P., Daniilidis, A., Dafopoulos, K., Botsis, D., Vrachnis, N., 2012. Endocrine, paracrine, and autocrine placental mediators in labor. Hormones (Athens) 11, 397–409.
Janszen, B.P.M., Bevers, M.M., van Tol, H.T.M., Dieleman, S.J., van der Weijden, G.C., Taverne, M.A.M., 1995. Oestrogen sulphatase activity in endometrium and foetal membranes of late gestational and parturient cows. Anim. Reprod. Sci. 37, 251–256.
Jones, J.I., Clemmons, D.R., 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev. 16, 3–34.
Jones, R.L., Stoikos, C., Findlay, J.K., Salamonsen, L.A., 2006. TGF-beta superfamily expression and actions in the endometrium and placenta. Reproduction 132, 217–232.
Karalis, K., Goodwin, G., Majzoub, J.A., 1996. Cortisol blockade of progesterone: a possible molecular mechanism involved in the initiation of human labor. Nat. Med. 2, 556–560.
Kautz, E., Gram, A., Aslan, S., Ay, S.S., Selcuk, M., Kanca, H., Koldas, E., Akal, E., Karakas, K., Findik, M., Boos, A., Kowalewski, M.P., 2014. Expression of genes involved in the embryo-maternal interaction in the early-pregnant canine uterus. Reproduction 147, 703–717.

Kota, S.K., Gayatri, K., Jammula, S., Kota, S.K., Krishna, S.V., Meher, L.K., Modi, K.D., 2013. Endocrinology of parturition. Indian J. Endocrinol. Metab. 17, 50–59.
Kowalewski, M.P., 2014. Luteal regression vs. prepartum luteolysis: regulatory mechanisms governing canine corpus luteum function. Reprod. Biol. 14, 89–102.
Kowalewski, M.P., 2018. Selected Comparative Aspects of Canine Female Reproductive Physiology. In: Encyclopedia of Reproduction, pp. 682–691.
Kowalewski, M.P., Beceriklisoy, H.B., Aslan, S., Agaoglu, A.R., Hoffmann, B., 2009. Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch. Anim. Reprod. Sci. 116, 129–138.
Kowalewski, M.P., Beceriklisoy, H.B., Pfarrer, C., Aslan, S., Kindahl, H., Kucukaslan, I., Hoffmann, B., 2010. Canine placenta: a source of prepartal prostaglandins during normal and antiprogestin-induced parturition. Reproduction 139, 655–664.
Kowalewski, M.P., Gram, A., Kautz, E., Graubner, F.R., 2015. The dog: nonconformist, not only in maternal recognition signaling. Adv. Anat. Embryol. Cell Biol. 216, 215–237.
Kowalewski, M.P., Meyer, A., Hoffmann, B., Aslan, S., Boos, A., 2011. Expression and functional implications of peroxisome proliferator-activated receptor gamma (PPARgamma) in canine reproductive tissues during normal pregnancy and parturition and at antiprogestin induced abortion. Theriogenology 75, 877–886.
Kowalewski, M.P., Schuler, G., Taubert, A., Engel, E., Hoffmann, B., 2006. Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. Theriogenology 66, 1423–1430.
Kubiczkova, L., Sedlarikova, L., Hajek, R., Sevcikova, S., 2012. TGF-beta - an excellent servant but a bad master. J. Transl. Med. 10, 183.
Lauszus, F.F., Fuglsang, J., 2016. IGF-1 is associated with fetal growth and preterm delivery in type 1 diabetic pregnancy. Gynecol. Endocrinol. 32, 488–491.
Liggins, G.C., Fairclough, R.J., Grieves, S.A., Kendall, J.Z., Knox, B.S., 1973. The mechanism of initiation of parturition in the ewe. Recent Prog. Horm. Res. 29, 111–159.
Lockwood, C.J., 2004. The initiation of parturition at term. Obstet. Gynecol. Clin. North Am. 31 (935–947), xii.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.
Macias, H., Hinck, L., 2012. Mammary gland development. Wiley Interdiscip. Rev. Dev. Biol. 1, 533–557.
Magyar, D.M., Fridshal, D., Elsner, C.W., Glatz, T., Eliot, J., Klein, A.H., Lowe, K.C., Buster, J.E., Nathanielsz, P.W., 1980. Time-trend analysis of plasma cortisol concentrations in the fetal sheep in relation to parturition. Endocrinology 107, 155–159.
Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N., Daly, T.J., Davis, S., Sato, T.N., Yancopoulos, G.D., 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277, 55–60.
Mark, P.J., Augustus, S., Lewis, J.L., Hewitt, D.P., Waddell, B.J., 2009. Changes in the placental glucocorticoid barrier during rat pregnancy: impact on placental corticosterone levels and regulation by progesterone. Biol. Reprod. 80, 1209–1215.
Maroni, D., Davis, J.S., 2011. TGFB1 disrupts the angiogenic potential of microvascular endothelial cells of the corpus luteum. J. Cell Sci. 124, 2501–2510.
Maroni, D., Davis, J.S., 2012. Transforming growth factor Beta 1 stimulates profibrotic activities of luteal fibroblasts in cows. Biol. Reprod. 87, 127.
Mason, J.I., France, J.T., Magness, R.R., Murry, B.A., Rosenfeld, C.R., 1989. Ovine placental steroid 17 alpha-hydroxylase/C-17,20-lyase, aromatase and sulphatase in dexamethasone-induced and natural parturition. J. Endocrinol. 122, 351–359.
Massague, J., 2012. TGFbeta signalling in context. Nat. Rev. Mol. Cell Biol. 13, 616–630.
Matthews, S.G., Challis, J.R., 1996. Regulation of the hypothalamo-pituitary-adrenocortical axis in fetal sheep. Trends Endocrinol. Metab. 7, 239–246.
Maymon, E., Romero, R., Pacora, P., Gervasi, M.T., Bianco, K., Ghezzi, F., Yoon, B.H., 2000. Evidence for the participation of interstitial collagenase (matrix metalloproteinase 1) in preterm premature rupture of membranes. Am. J. Obstet. Gynecol. 183, 914–920.
Meier, S., Lau, T.M., Jenkin, G., Fairclough, R.J., 1995. Oxytocin-induced prostaglandin F2 alpha release and endometrial oxytocin receptor concentrations throughout pregnancy in ewes. J. Reprod. Fertil. 103, 233–238.
Mi, H., Huang, X., Muruganujan, A., Tang, H., Mills, C., Kang, D., Thomas, P.D., 2017. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucl. Acids Res. 45, D183–d189.
Murphy, B.E., Clark, S.J., Donald, I.R., Pinsky, M., Vedady, D., 1974. Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. Am. J. Obstet. Gynecol. 118, 538–541.
Nathanielsz, P.W., 1998. Comparative studies on the initiation of labor. Eur. J. Obstet. Gynecol. Reprod. Biol. 78, 127–132.
Nishiyama, T., Tsumagari, S., Ito, M., Kimura, J., Watanabe, G., Taya, K., Takeishi, M., 1999. Immunohistochemical study of steroidogenic enzymes in the ovary and placenta during pregnancy in the dog. Anat. Histol. Embryol. 28, 125–129.
Nnamani, M.C., Plaza, S., Romero, R., Wagner, G.P., 2013. Evidence for independent evolution of functional progesterone withdrawal in primates and guinea pigs. Evol. Med. Public Health 2013, 273–288.
Nohr, B., Hoffmann, B., Steinetz, B.E., 1993. Investigation of the endocrine control of parturition in the dog by application of an antigestagen. J. Reprod. Fertil. Suppl. 47, 542–543.
Nowak, M., Gram, A., Boos, A., Aslan, S., Ay, S.S., Onyay, F., Kowalewski, M.P., 2017. Functional implications of the utero-placental relaxin (RLN) system in the dog throughout pregnancy and at term. Reproduction 154, 415–431.
O'Connor, A.E., McFarlane, J.R., Hayward, S., Yohkaichiya, T., Groome, N.P., de Kretser, D.M., 1999. Serum activin A and follistatin concentrations during human pregnancy: a cross-sectional and longitudinal study. Hum. Reprod. 14, 827–832.
Olson, D.M., 2003. The role of prostaglandins in the initiation of parturition. Best Pract. Res. Clin. Obstet. Gynaecol. 17, 717–730.
Pettersson, C.H., Tidholm, A., 2009. Safety and efficacy of mid-term pregnancy termination using aglepristone in dogs. J. Small Anim. Pract. 50, 120–123.
Qi, W., Schlapbach, R., Rehrauer, H., 2017. RNA-seq data analysis: from raw data quality control to differential expression analysis. Methods Mol. Biol. 1669, 295–307.
Ramos, T.N., Bullard, D.C., Barnum, S.R., 2014. ICAM-1: isoforms and phenotypes. J. Immunol. 192, 4469–4474.
Rigau, T., Rodriguez-Gil, J.E., Garcia, F., del Alamo, M.M., 2011. Partial foetal retention following aglepristone treatment in a bitch. Reprod. Domest. Anim. 46, 738–741.
Robertson, H.A., King, G.J., 1974. Plasma concentrations of progesterone, oestrone, oestradiol-17beta and of oestrone sulphate in the pig at implantation, during pregnancy and at parturition. J. Reprod. Fertil. 40, 133–141.
Schneider, M.R., Wolf, E., Hoeflich, A., Lahm, H., 2002. IGF-binding protein-5: flexible player in the IGF system and effector on its own. J. Endocrinol. 172, 423–440.
Schuler, G., Hartung, F., Hoffmann, B., 1994. Investigations on the use of C-21-steroids as precursors for placental oestrogen synthesis in the cow. Exp. Clin. Endocrinol. 102, 169–174.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504.
Shenavai, S., Preissing, S., Hoffmann, B., Dilly, M., Pfarrer, C., Ozalp, G.R., Caliskan, C., Seyrek-Intas, K., Schuler, G., 2012. Investigations into the mechanisms controlling parturition in cattle. Reproduction 144, 279–292.
Steele, P.A., Flint, A.P., Turnbull, A.C., 1976. Activity of steroid C-17,20 lyase in the ovine placenta: effect of exposure to foetal glucocorticoid. J. Endocrinol. 69, 239–246.
Steiger, K., Politt, E., Hoeftmann, T., Meyer-Lindenberg, A., Schoon, H.A., Gunzel-Apel, A.R., 2006. Morphology of canine placental sites after induced embryonic or fetal death. Theriogenology 66, 1709–1714.
Sun, K., Adamson, S.L., Yang, K., Challis, J.R., 1999. Interconversion of cortisol and cortisone by 11beta-hydroxysteroid dehydrogenases type 1 and 2 in the perfused human placenta. Placenta 20, 13–19.
Sun, K., Yang, K., Challis, J.R., 1998. Glucocorticoid actions and metabolism in pregnancy: implications for placental function and fetal cardiovascular activity. Placenta 19, 353–360.
Ulick, S., Ramirez, L.C., New, M.I., 1977. An abnormality in steroid reductive metabolism in a hypertensive syndrome. J. Clin. Endocrinol. Metab. 44, 799–802.
Vermeirsch, H., Simoens, P., Lauwers, H., 2000. Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth. Anat. Rec. 260, 42–50.
Veronesi, M.C., Battocchio, M., Marinelli, L., Faustini, M., Kindahl, H., Cairoli, F., 2002. Correlations among body temperature, plasma progesterone, cortisol and prostaglandin F2alpha of the periparturient bitch. J. Vet. Med. A Physiol. Pathol. Clin. Med. 49, 264–268.
Verstegen-Onclin, K., Verstegen, J., 2008. Endocrinology of pregnancy in the dog: a review. Theriogenology 70, 291–299.
Wanke, M., Loza, M.E., Monachesi, N., Concannon, P., 1997. Clinical use of dexamethasone for termination of unwanted pregnancy in dogs. J. Reprod. Fertil. Suppl. 51, 233–238.
Wiesli, P., Zwimpfer, C., Zapf, J., Schmid, C., 2006. Pregnancy-induced changes in insulin-like growth factor I (IGF-I), insulin-like growth factor binding protein 3 (IGFBP-3), and acid-labile subunit (ALS) in patients with growth hormone (GH) deficiency and excess. Acta Obstet. Gynecol. Scand. 85, 900–905.
Wijayarathna, R., de Kretser, D.M., 2016. Activins in reproductive biology and beyond. Hum. Reprod. Update 22.
Wu, W.X., Verbalis, J.G., Hoffman, G.E., Derks, J.B., Nathanielsz, P.W., 1996. Characterization of oxytocin receptor expression and distribution in the pregnant sheep uterus. Endocrinology 137, 722–728.
Xie, F., Xiao, P., Chen, D., Xu, L., Zhang, B., 2012. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol. Biol.
Yang, L., Froio, R.M., Sciuto, T.E., Dvorak, A.M., Alon, R., Luscinskas, F.W., 2005. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. Blood 106, 584–592.
Young, M.D., Wakefield, M.J., Smyth, G.K., Oshlack, A., 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 11, R14.
Yu, L., Li, D., Liao, Q.P., Yang, H.X., Cao, B., Fu, G., Ye, G., Bai, Y., Wang, H., Cui, N., Liu, M., Li, Y.X., Li, J., Peng, C., Wang, Y.L., 2012. High levels of activin A detected in preeclamptic placenta induce trophoblast cell apoptosis by promoting nodal signaling. J. Clin. Endocrinol. Metab. 97, E1370–1379.
Zakar, T., Hertelendy, F., 2007. Progesterone withdrawal: key to parturition. Am. J. Obstet. Gynecol. 196, 289–296.
Zatta, S., Rehrauer, H., Gram, A., Boos, A., Kowalewski, M.P., 2017. Transcriptome analysis reveals differences in mechanisms regulating cessation of luteal function in pregnant and non-pregnant dogs. BMC Genomics 18, 757.
Zone, M., Wanke, M., Rebuelto, M., Loza, M., Mestre, J., Duchene, A., Concannon, P., 1995. Termination of pregnancy in dogs by oral administration of dexamethasone. Theriogenology 43, 487–494.